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Genetic background of uropathogenic *Escherichia coli* isolates from Slovenia in relation to fluoroquinolone and sulfamethoxazole/trimethoprim resistance

Genetsko ozadje uropatogenih sevov bakterije *Escherichia coli* iz Slovenije v povezavi z odpornostjo proti fluorokinolonom in sulfametoksazol/trimetoprimu

[1]Marjanca Starčič Erjavec, [2]Anja Palandačić, [1]Darja Žgur-Bertok, [1]Jerneja Ambrožič Avguštin*

[1]Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, SI-1000 Ljubljana, Slovenia
[2]Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3, 1230 Domžale, Slovenia
* correspondence: jerneja.ambrozic@bf.uni-lj.si

Abstract: A total of 99 *E. coli* urinary tract isolates were investigated for phylogenetic groups and 21 virulence related genes in relation to fluoroquinolone and sulfamethoxazole/trimethoprim resistance. We found that the B2 group was by far the most prevalent among susceptible isolates, while resistant isolates were more evenly distributed among groups A, B2 and D. Isolates from the B2 group exhibited the highest prevalence of virulence factors. Virulence genes *hlyA*, *iroN* and *kpsMTII* were statistically associated with fluoroquinolone susceptible isolates and *picU* with sulfamethoxazole/trimethoprim susceptible isolates. Fluoroquinolone susceptible isolates of the phylogenetic group A were significantly associated with genes *papGII*, *kpsMTII* and *iss* and the susceptible group B_2 isolates with genes *hra* in *iroN*. Among isolates susceptible to sulfamethoxazole/trimethoprim the presence of the *hra* gene was statistically significantly associated with phylogenetic group B2, while among resistant isolates, *papGII* was associated with phylogenetic group D.

Keywords: *Escherichia coli*, urinary tract, phylogenetic groups, virulence trait, fluoroquinolone resistance, sulfamethoxazole/trimethoprim resistance

Izvleček: V naši raziskavi smo 99 uropatogenih izolatov *E. coli* uvrstili v filogenetske skupine in pri vsakem preverili prisotnost 21-ih genov povezanih z virulenco ter podatke analizirali v povezavi z odpornostjo izolata za fluorokinolone in sulfametoksazol/trimetoprim. Ugotovili smo, da se izolati, ki so občutljivi za fluorokinolone in /ali sulfametoksazol/trimetoprim uvrščajo predvsem v filogenetsko skupino B2, odporni izolati pa v približno enakih deležih v skupine A, B2 in D. Izolati v filogenetski skupini B2 so imeli največ genskih zapisov za virulentne dejavnike. Izolati občutljivi za fluorokinolone so imeli statistično značilno pogosteje preučevane genske zapise *hlyA*, *iroN* in *kpsMTII* v primerjavi z odpornimi izolati, medtem ko so imeli izolati občutljivi za sulfametoksazol/trimetoprim v primerjavi z odpornimi izolati statistično značilno pogosteje genski zapis za *picU*. Pri izolatih, ki so bili občutljivi za fluorokinolone, smo ugotovili statistično značilne povezave med prisotnostjo genov *papGII*, *kpsMTII* ter *iss* in uvrstitvijo izolata v filogenetsko skupino A ter genov *hra* in *iroN* ter uvrstitvijo

izolata v filogenetsko skupino B2. Pri izolatih, ki so bili občutljivi za sulfametoksazol/trimetoprim, je bila statistično značilna povezava med prisotnostjo gena *hra* in uvrstitvijo izolata v filogenetsko skupino B2, pri odpornih izolatih pa je bil gen *papGII* statistično značilno povezan z uvrstitvijo izolata v filogenetsko skupino D.

Ključne besede: *Escherichia coli*, sečila, filogenetske skupine, virulentni dejavniki, odpornost proti fluorokinolonom, odpornost proti trimetoprimu in sulfametoksazolu

Introduction

Urinary tract infections (UTIs) are one of the most frequent infectious diseases encountered in the developed world. Uropathogenic *Escherichia coli* (*E. coli*) strains (UPEC) are the major cause of uncomplicated UTI worldwide. In Slovenia *E. coli* causes approximately 80% of all UTIs (Lindič 2005). In comparison to commensal *E. coli* strains, UPEC possess an array of virulence factors namely, adhesins, toxins, polysaccharide coatings, invasins, iron uptake systems and systems to evade the host immune response (Oelschlaeger et al. 2002). UPEC mainly belong to the B2 phylogenetic group and to a lesser extent to the D group, while commensal strains belong to groups A and B1 (Picard et al. 1999). The most frequently prescribed drugs for the treatment of UTIs in general practices in Slovenia are trimethoprim/sulfamethoxazole (57% of prescribed antibiotics) and the fluoroquinolones norfloxacin and ciprofloxacin (38% of prescribed antibiotics) (Car et al. 2003). However, a major problem in treatment of UTIs is the emergence of *E. coli* strains resistant to these first-line antimicrobials. To unravel the relationship between resistance and virulence, several studies have dealt with the characteristics of fluoroquinolone and/or trimethoprim/sulfamethoxazole resistant strains, including phylogenetic background (A, B1, B2 and D group) and virulence factors (Drews et al. 2005, Horcajada et al. 2005, Johnson et al. 2003, Johnson et al. 2005, Johnson et al. 2009, Moreno et al. 2006, Piatti et al. 2008, Takahashi et al. 2009, Vila et al. 2002). Since no comparable data are available for UPEC isolates from Slovenia, we investigated the distribution of virulence genes among phylogenetic groups in relation to fluoroquinolone and sulfamethoxazole/trimethoprim resistance.

Material and methods

Bacterial strains

The UPEC isolates investigated in this study were collected and identified at the Institute of Public Health of the Republic of Slovenia (IVZ) between the years 2004–2007. The strains were isolated from urine of outpatients with cystitis, who sought help at general practises in Slovenian Community Health Centres. Only one isolate per patient was included in our study. A random sample of 45 ciprofloxacin resistant and 54 ciprofloxacin susceptible isolates, as determined by the disk diffusion method and interpreted according to the CLSI standards (Clinical and Laboratory Standards Institute 2007), were included in the study. Additionally all isolates were also tested for sulfamethoxazole/trimethoprim resistance.

Detection of phylogenetic groups and virulence factors

DNA to be PCR amplified for detection of phylogenetic groups and virulence factors was released from whole bacterial cells by boiling according to Le Bouguenec et al. (1992). For all isolates the phylogenetic groups (A, B1, B2 and D) were determined using the triplex PCR described by Clermont et al. (2000). Further, all isolates were screened for the presence of 21 urovirulence genes, including fimbriae/adhesins (*fimH* – type 1-fimbrial adhesin, *papGII* – P-fimbrial adhesin II, *sfaDE* – S-fimbriae, *bmaE* – M-fimbrial adhesin, *gafD* – G-fimbrial adhesin, *iha* – non fimbrial adhesin Iha and *hra* – non fimbrial adhesin Hra), toxins/autotransporters (*hlyA* – hemolysin A, *hbp* – haemoglobin protease, *sat* – secreted autotransporter toxin, *vat* – vacuolating autotransporter toxin and *picU* – autotransporter involved in intestinal colonization PicU), invasins (*ompA* – outer membrane

protein A, *ibeA* – invasion of brain endothelium and *aslA* – arylsulphatase-like protein), genes involved in iron acquisition (*iucD* – aerobactin synthesis, *iroN* – catecholate siderophore receptor and *irp2* – yersiniabactin biosynthesis), capsule synthesis (*kpsMTII*), increased serum survival (*iss*) and uropathogenic specific protein (*usp*). The employed primers are available at http://www.bf.uni-lj.si/fileadmin/users/1/biologija/genetika/Table-PCR-primers.pdf.

Statistical analysis

The significance of the results was established using the Fisher's exact test (2-tailed) available on-line on the web site http://www.langsrud.com/fisher.htm. The threshold for statistical significance was set at a P value < 0.05.

Results

Prevalence of phylogenetic groups and virulence factors in relation to resistance phenotypes

As seen from Table 1, the majority (30 out of 54, 56%) of the fluoroquinolone-susceptible strains were assigned to the phylogenetic group B2, followed by the D group with 17 strains (31%). The fluoroquinolone-resistant strains were evenly distributed among the phylogenetic groups A, B2 and D; 13 strains (29%) belonged to the A group, 14 strains (31%) to the B2 group and 16 strains (35%) to the D group. The differences between the prevalence of fluoroquinolone-susceptible and resistant strains in the A and B2 groups were statistically significant. Of the examined virulence genes 13 occurred with a higher prevalence among fluoroquinolone susceptible isolates while the associations of *hlyA*, *iroN* and *kpsMTII* with susceptibility were statistically significant. In accordance with the higher prevalence of the majority of virulence genes, the average virulence score among susceptible strains was higher compared to resistant strains (7.76 versus 6.13). The majority (26 out of 48, 54%) of the sulfamethoxazole/trimethoprim-susceptible strains were assigned to the phylogenetic group B2, followed by the D group with 15 strains (31%). The sulfamethoxazole/trimethoprim-resistant strains were distributed more evenly among the phylogenetic groups A (25%), B2 (35%) and D (35%). However, the differences between the number of sulfamethoxazole/trimethoprim-susceptible and resistant strains with regard to phylogenetic group was statistically not significant. Twelve of the examined virulence genes occurred with a higher prevalence among sulfamethoxazole/trimethoprim-susceptible isolates (Table 1), but only the occurrence of *picU* was statistically significant. The average virulence score among sulfamethoxazole/trimethoprim susceptible and resistant isolates was 7.31 versus 6.75.

Significant associations of virulence genes and phylogenetic groups in relation to resistance phenotypes

Only virulence genes with a prevalence of $\geq 10\%$ were selected for the analysis of associations of virulence genes and phylogenetic groups in relation to resistance phenotypes (Table 2).

While among fluoroquinolone and sulfamethoxazole/trimethoprim-susceptible strains the majority of the virulence genes were detected in isolates of the B2 group, only the occurrence of *hra* and *iroN* was statistically significant, the first among fluoroquinolone and sulfamethoxazole/trimethoprim-susceptible isolates and the second only among sulfamethoxazole/trimethoprim-susceptible isolates (Table 2). Among the latter isolates *papGII, kpsMTII* and *iss* were significantly associated with phylogenetic group A. Among sulfamethoxazole/trimethoprim-resistant isolates only *papGII* was significantly associated with the D group isolates (Table 2).

Discussion

This study showed that 56% of fluoroquinolone-susceptible and 54% of sulfamethoxazole/trimethoprim-susceptible isolates belonged to phylogenetic group B2, while fluoroquinolone- and sulfamethoxazole/trimethoprim-resistant isolates were evenly distributed among groups A, B2, and D. However, the percentage of fluoroquinolone-resistant isolates belonging to group B2 has been increasing in the last decade. Studies on *E. coli* isolated from 1998 to 2003 reported 12% (Johnson

Table 1: Prevalence of phylogenetic groups and virulence genes in relation to resistance phenotypes among the studied *E. coli* isolates

Tabela 1: Prevalence filogenetskih skupin in genov za dejavnike virulence pri odpornih in občutljivih izolatih *E.coli*

	Prevalence [N (%)]					
	FQ			SXT		
	S	R	P	S	R	P
	(N = 54)	(N = 45)		(N = 48)	(N = 51)	
Phylogenetic group						
A	4 (7)	13 (29)	0.007	4 (8)	13 (25)	ns
B1	3 (6)	2 (4)	ns	3 (6)	2 (4)	ns
B2	30 (56)	14 (31)	0.016	26 (54)	18 (35)	ns
D	17 (31)	16 (35)	ns	15 (31)	18 (35)	ns
Virulence gene						
fimH	52 (96)	45 (100)	ns	47 (98)	50 (98)	ns
papGII	25 (46)	17 (38)	ns	19 (40)	23 (45)	ns
sfaDE	20 (37)	10 (22)	ns	19 (40)	11 (22)	ns
bmaE	2 (4)	0 (0)	ns	0 (0)	2 (4)	ns
gafD	0 (0)	0 (0)	ns	0 (0)	0 (0)	ns
iha	21 (39)	18 (40)	ns	14 (29)	25 (49)	ns
hra	16 (30)	7 (16)	ns	14 (29)	9 (18)	ns
hlyA	10 (19)	0 (0)	0.002	6 (13)	4 (8)	ns
hbp	2 (4)	3 (7)	ns	3 (6)	2 (4)	ns
sat	14 (26)	11 (24)	ns	11 (23)	14 (27)	ns
vat	6 (11)	2 (4)	ns	6 (13)	2 (4)	ns
picU	14 (26)	5 (11)	ns	15 (31)	4 (8)	0.005
ompA	49 (91)	41 (91)	ns	43 (90)	47 (92)	ns
ibeA	8 (15)	8 (18)	ns	8 (17)	8 (16)	ns
aslA	0 (0)	0 (0)	ns	0 (0)	0 (0)	ns
iucD	32 (59)	29 (64)	ns	25 (52)	36 (71)	ns
iroN	37 (69)	17 (38)	0.003	29 (60)	25 (49)	ns
irp2	44 (81)	30 (67)	ns	40 (83)	34 (67)	ns
kpsMTII	39 (72)	19 (42)	0.004	32 (67)	26 (51)	ns
iss	12 (22)	8 (18)	ns	7 (15)	13 (25)	ns
usp	16 (30)	6 (13)	ns	13 (27)	9 (18)	ns
AVS	7.76	6.13		7.31	6.75	

FQ: Fluoroquinolone; Sxt: Sulfamethoxazole/Trimethoprim; S: Susceptible; R: Resistant; AVS: average virulence score (the average virulence score was calculated as the sum of all detected virulence associated genes divided with the number of isolates per group)/; ns – not statistically significant

Table 2: Associations of virulence genes with phylogenetic groups in relation to resistance phenotypes among the studied *E. coli* isolates.
Tabela 2: Povezava genov z zapisom za dejavnike virulence z uvrstitvijo v filogenetsko skupino in odpornostjo.

		Prevalence [no. (%)]									
		FQ				SXT					
		S		R		**P**	S		R		**P**
Virulence gene	Phylogenetic group										
fimH	A	4	(100)	13	(100)	ns	4	(100)	13	(100)	ns
	B1	3	(100)	2	(100)	ns	3	(100)	2	(100)	ns
	$B2_3$	29	(97)	14	(100)	ns	26	(100)	17	(94)	ns
	D	16	(94)	16	(100)	ns	14	(93)	18	(100)	ns
papGII	A	3	(75)	0	(0)	0,006	2	(50)	1	(8)	ns
	B1	1	(33)	0	(0)	ns	1	(33)	0	(0)	ns
	$B2_3$	13	(43)	5	(36)	ns	11	(42)	7	(39)	ns
	D	8	(47)	12	(75)	ns	5	(33)	15	(83)	0,005
sfaDE	A	1	(25)	1	(100)	ns	0	(0)	2	(15)	ns
	B1	0	(0)	0	(0)	ns	0	(0)	0	(0)	ns
	$B2_3$	15	(50)	6	(43)	ns	15	(58)	6	(33)	ns
	D	4	(24)	3	(19)	ns	4	(27)	3	(21)	ns
bmaE	A	0	(0)	0	(0)	ns	0	(0)	0	(0)	ns
	B1	1	(33)	0	(0)	ns	0	(0)	1	(50)	ns
	$B2_3$	0	(0)	0	(0)	ns	0	(0)	0	(0)	ns
	D	1	(11)	0	(0)	ns	0	(0)	1	(7)	ns
iha	A	1	(25)	4	(33)	ns	0	(0)	5	(42)	ns
	B1	0	(0)	0	(0)	ns	0	(0)	0	(0)	ns
	$B2_3$	12	(40)	8	(57)	ns	9	(35)	11	(61)	ns
	D	8	(47)	6	(38)	ns	5	(33)	9	(50)	ns
hra	A	0	(0)	4	(33)	ns	0	(0)	4	(33)	ns
	B1	0	(0)	0	(0)	ns	0	(0)	0	(0)	ns
	$B2_3$	13	(43)	1	(7)	0,019	12	(46)	2	(11)	0,021
	D	3	(18)	2	(14)	ns	2	(33)	3	(21)	ns
hlyA	A	0	(0)	0	(0)	ns	0	(0)	0	(0)	ns
	B1	0	(0)	0	(0)	ns	0	(0)	0	(0)	ns
	$B2_3$	7	(23)	0	(0)	ns	4	(15)	3	(17)	ns
	D	3	(38)	0	(0)	ns	2	(33)	1	(25)	ns
hbp	A	0	(0)	1	(8)	ns	1	(25)	0	(0)	ns
	B1	0	(0)	1	(50)	ns	0	(0)	1	(50)	ns
	$B2_3$	2	(7)	0	(0)	ns	1	(4)	1	(6)	ns
	D	0	(0)	1	(7)	ns	1	(11)	0	(0)	ns
sat	A	1	(25)	0	(0)	ns	0	(0)	1	(8)	ns
	B1	0	(0)	0	(0)	ns	0	(0)	0	(0)	ns
	$B2_3$	8	(27)	7	(50)	ns	7	(27)	8	(44)	ns
	D	5	(29)	4	(29)	ns	4	(44)	5	(28)	ns

		Prevalence [no. (%)]								
		FQ				SXT				
		S		R		**P**	S		R		P
Virulence gene	Phylogenetic group									
vat	A	0	(0)	1	(8)	ns	0	(0)	1	(8)	ns
	B1	0	(0)	0	(0)	ns	0	(0)	0	(0)	ns
	$B2_3$	6	(20)	1	(7)	ns	6	(23)	1	(6)	ns
	D	0	(0)	0	(0)	ns	0	(0)	0	(0)	ns
picU	A	2	(50)	1	(8)	ns	2	(50)	1	(8)	ns
	B1	0	(0)	0	(0)	ns	0	(0)	0	(0)	ns
	$B2_3$	9	(30)	4	(29)	ns	10	(38)	3	(17)	ns
	D	3	(18)	0	(0)	ns	3	(20)	0	(0)	ns
ompA	A	3	(75)	10	(77)	ns	2	(50)	11	(85)	ns
	B1	1	(33)	1	(50)	ns	1	(33)	1	(50)	ns
	$B2_3$	28	(93)	14	(100)	ns	25	(96)	17	(96)	ns
	D	17	(100)	16	(100)	ns	15	(100)	18	(100)	ns
ibeA	A	0	(0)	1	(8)	ns	0	(0)	1	(8)	ns
	B1	0	(0)	2	(100)	ns	1	(33)	1	(50)	ns
	$B2_3$	7	(23)	2	(14)	ns	5	(19)	4	(22)	ns
	D	1	(11)	3	(21)	ns	2	(22)	2	(14)	ns
iucD	A	4	(100)	7	(54)	ns	3	(75)	8	(62)	ns
	B1	2	(67)	1	(50)	ns	1	(33)	2	(100)	ns
	$B2_3$	17	(57)	12	(86)	ns	15	(58)	14	(78)	ns
	D	9	(53)	9	(56)	ns	6	(40)	12	(67)	ns
iroN	A	3	(75)	4	(31)	ns	2	(50)	5	(38)	ns
	B1	2	(67)	1	(50)	ns	1	(33)	2	(100)	ns
	$B2_3$	25	(83)	7	(50)	0,032	21	(81)	11	(61)	ns
	D	7	(41)	5	(36)	ns	5	(33)	7	(39)	ns
irp2	A	4	(100)	5	(42)	ns	4	(100)	5	(42)	ns
	B1	2	(67)	1	(50)	ns	1	(33)	2	(100)	ns
	$B2_3$	27	(90)	14	(100)	ns	24	(92)	17	(94)	ns
	D	11	(65)	10	(63)	ns	11	(73)	10	(56)	ns
kpsMTII	A	2	(50)	0	(100)	0,044	0	(0)	2	(17)	ns
	B1	0	(0)	1	(50)	ns	0	(0)	1	(50)	ns
	$B2_3$	24	(80)	8	(57)	ns	20	(77)	12	(67)	ns
	D	13	(76)	10	(63)	ns	12	(80)	11	(61)	ns
iss	A	4	(100)	2	(17)	0,006	3	(75)	3	(25)	ns
	B1	1	(33)	0	(0)	ns	0	(0)	1	(50)	ns
	$B2_3$	6	(20)	1	(7)	ns	3	(12)	4	(22)	ns
	D	1	(12)	5	(36)	ns	1	(11)	5	(28)	ns

		Prevalence [no. (%)]							
		FQ				SXT			
		S		R	**P**	S		R	P
Virulence gene	Phylogenetic group								
usp	A	1	(25)	0	(0) ns	0	(0)	1 (8)	ns
	B1	0	(0)	0	(0) ns	0	(0)	0 (0)	ns
	$B2_3$	13	(43)	6	(43) ns	11	(42)	8 (44)	ns
	D	2	(25)	0	(0) ns	2	(33)	0 (0)	ns

Virulence traits with ≥10 % prevalence were included.
FQ: Fluoroquinolone; SXT: sulfamethoxazole/trimethoprim; S: Susceptible; R: Resistant;

et al. 2003), 0% (Johnson et al. 2005) and 11% (Moreno et al. 2006) of group B2 fluoroquinolone resistant isolates, while among *E. coli* isolated from 2005 to 2007 the percentage raised upon 50% (Takahashi 2009) and 49% (Johnson et al. 2009). The relatively high percentage (31%) of fluoroquinolone-resistant isolates belonging to group B2 in our collection, comprising *E. coli* isolates from 2004–2007, is therefore in agreement with this trend.

The prevalence of virulence genes among the studied UPEC isolates from Slovenia revealed that, resistant isolates possessed less virulence genes than susceptible isolates and vice versa, which is in accordance with results of similar studies from other countries (Johnson et al. 2003, Johnson et al. 2005, Piatti et al. 2008). However, the examined UPEC collections exhibited distinct significant associations of fluoroquinolone and sulfamethoxazole/trimethoprim resistance pattern with particular virulence genes. For example, in our study we found that, the virulence genes *hra* and *iroN* were statistically significant among fluoroquinolone- susceptible phylogenetic group B isolates, while Piatti et al. (2008) reported a significant association between gene *iss* and fluoroquinolone susceptible group B isolates.

To elucidate the basis of such differences further studies are needed, as for now we can only speculate that sample size, the characteristics of the studied isolates and their hosts might be relevant. In addition, the evolutionary origin of the association between possession of virulence factors and susceptibility needs to be clarified. To this end several hypothesis have been postulated: (i) acquisition/loss of pathogenicity islands, (ii) incompatibility of plasmids encoding virulence and resistance, (iii) less virulent strains are more prone to acquire resistance, (iv) acquisition of resistance promotes loss of virulence.

To summarise, our results in comparison to studies performed before 2004 show a steep increase in the prevalence of fluoroquinolone-resistant strains belonging to the B2 group. It is of great concern that *E. coli* strains of the B2 group, which are known to exhibit the greatest virulence potential, are readily acquiring resistance to fluoroquinolones. These strains addtionally equipped with CTX-M plasmids carrying extended-spectrum beta-lactamases (ESBL) and plasmid-mediated quinolone resistance (PMQR) genes might be the source of highly resistant and virulent clonal groups, such as *E. coli* ST131.

Povzetek

Bakterije vrste *Escherichia coli* so sicer del normalne črevesne mikrobiote ljudi in živali s stalno telesno temperaturo, vendar med njimi obstajajo tudi (potencialno) patogeni sevi, ki lahko povzročijo različne okužbe. Od komenzalnih sevov se običajno ločijo po prisotnosti številnih genov z zapisi za dejavnike virulence, ki bakterijam med drugim omogočajo pritrjanje na gostiteljske celice, poškodbe gostiteljske celice, privzem železa in izogibanje imunskemu sistemu. Uropatogeni sevi *E. coli* so poglavitni povzročitelji nezapletenih okužb sečil. Za zdravljenje teh okužb se najpogosteje uporabljajo protimikrobne snovi sulfametoksazol/trimetoprim in fluorokinoloni vendar je zaradi naraščanja odpornih sevov zdravljenje vse težje. Zato nas je zanimalo ali obstaja kakšna povezava med genetskim ozadjem uropatogenih

sevov in odpornostjo proti sulfametoksazol/trimetoprimu in fluorokinolonom. V našo raziskavo smo vključili 99, za protimikrobni učinkovini občutljivih in odpornih bakterij *E. coli*, ki so bile izolirane iz urina bolnikov z vnetjem sečil. Vse seve smo uvrstili v filogenetske skupine in jih pregledali za prisotnost 21-ih genov z zapisom za dejavnike virulence. Ugotovili smo, da je največ občutljivih izolatov iz filogenetske skupine B2, odporni izolati pa so enakomerno razporejeni v filogenetskih skupinah A, B2 in D in, da imajo izolati iz filogenetske skupini B2 največ genskih zapisov za virulentne dejavnike. Poleg tega smo izsledili nekatere statistično značilne povezave med prisotnostjo genskega zapisa za virulentne dejavnike, z uvrstitvijo seva v filogenetsko skupino in odpornostjo proti sulfametoksazol/trimetoprimu in/ali fluorokinolonom. Zaključki naše raziskave so, da so sevi z večjim naborom genov, ki pripomorejo k virulenci, bolj občuljivi za protimikrobni učinkovini in obratno ter, da so uropatogeni izolati *E. coli* iz Slovenije po svojem naboru genskih zapisov za dejavnike virulence podobni uropatogenim izolatom iz drugih geografskih okolij. Razlika je le v značilnih statističnih povezavah posameznih genov s filogenetskimi skupinami.

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Molecular modelling of FtsZ proteins based on their homology in *Escherichia coli* and *Mycobacterium tuberculosis* as the key stage of rational design of new antituberculous compounds

Molekularno modeliranje proteinov FtsZ na osnovi njihove homologije v *Escherichia coli* in *Mycobacterium tuberculosis* kot ključna stopnja racionalnega oblikovanja novih protituberkuloznih komponent

Oleh Demchuk [a], Pavel Karpov [a], Peter Raspor [b], Yaroslav Blume [a*]

[a]Department of Genomics and Molecular Biotechnology, Institute of Food Biotechnology and Genomics, Natl. Academy of Sciences of Ukraine, Osipovskogo str, 2a, Kyiv, 04123, Ukraine;
[b]Biotechnical Faculty, University of Ljubljana, Jamnikarjeva, 101, 1000 Ljubljana, Slovenia
*correspondence: yablume@univ.kiev.ua

Abstract: The analysis of the quality of X-ray structures from *Mycobacterium tuberculosis* FtsZ proteins, which are deposited in the ProteinDataBank, gave a possibility to select a 2Q1Y (Chain A) structure as a template for future *in silico* research. Also several spatial models of FtsZ protein from *Escherichia coli* were reconstructed with on-line servers »SWISS-MODEL Workspace« and I-TASSER, than the most appropriate structure was selected. Basing on complex bioinformatic study, the model, which was rebuilt by SwissModel server from 2Q1Y (chain A) template, was supposed as the most significant.

Keywords: FtsZ, *Escherichia coli*, *Mycobacterium tuberculosis*, 3D-structure modelling and verification, *in silico*

Izvleček: Analiza struktur proteinov FtsZ z X žarki iz *Mycobacterium tuberculosis* deponiranih v »ProteinDataBank« je dala možnost izbora strukture 2Q1Y (veriga A) kot matrice za nadaljno *in silico* raziskavo. Nekaj prostorskih modelov proteina FtsZ iz bakterije *Escherichia coli* je bilo rekonstruiranih na on-line serverju SwissModel in I-Tasser, kateremu je sledil izbor najprimernejše strukture. Na osnovi celovite bioinformacijske preverbe kaže, da je model narejen na platformi serverja SwissModel iz matrice 2Q1Y (veriga A) najbolj sprejemljiv za uporabo.

Ključne besede: FtsZ, *Escherichia coli*, *Mycobacterium tuberculosis*, 3D-strukturno modeliranje in preverjanje, *in silico*

Introduction

Tuberculosis is the leading cause of death in the world from a single infectious disease, claiming over three million lives each year (Huang et al. 2006). Furthermore, poor patient compliance and inadequate control programs have lead to the emergence of multidrug-resistant strains of *M. tuberculosis* (Raviglione 2000). Bacterial resistance to three or more 'second-line' antibiotics is classified as extremely drug-resistant tuberculosis. Therefore, there is an urgent need

for the development of new anti-tuberculosis drugs with novel mechanism of action(s), which are active against drug-resistant as well as drug-sensitive *M. tuberculosis* strains. FtsZ (Filamentous temperature-sensitive protein Z) is an essential cell division protein has been shown to be a bacterial homolog of the mammalian cytoskeleton protein tubulin (Kumar et al. 2010). Accordingly, FtsZ protein is a very promising target for new antimicrobial drug development, and especially compounds effective against drug-resistant *M. tuberculosis* strains (Kumar et al. 2011). It is the perspective target for such numerous and diverse groups of low molecular weight compounds as benzimidazoles (Ohashi et al. 1999, Kumar et al. 2011), naphthalenesulfonates (Yu and Margolin 1998), azithromycins (Margalit et al. 2004), ethyl carbamates (White et al. 2000, 2002), diterpenoid phenols (Jaiswal et al. 2007) etc. At the same time, modern rational design of new compounds with antibacterial activity is impossible without stage of virtual screening, with application of accurate three-dimensional models of target FtsZ-proteins. The last is very important due to FtsZ – tubulin structure and function similarity, places particularly high demands on quality of 3D-models used for *in silico* molecular docking and virtual screening.

At present, Worldwide ProteinDataBank (wwPDB – www.pdb.org: RCSB PDB (USA)/ PDBe (Europe)/PDBj (Japan)) (Berman et al. 2003) contain the row of bacterial FtsZ X-ray structures of different resolution: 2R6R (1.70 Å) (Oliva et al. 2007) and 2R75 (1.40 Å) (Läppchen et al. 2008) from *Aquifex aeolicus*; 2VAM (2.50 Å) (Oliva et al. 2007), 2VXY (1.70 Å) (Haydon et al. 2008), 2RHH (2.00Å), 2RHJ (1.76 Å), 2RHL (2.45 Å) and 2RHO (2.45 Å) (Raymond et al. 2009) from *Bacillus subtilis*; 1FSZ (2.80 Å) (Löwe and Amos 1998), 1W5B (2.20 Å), 1W5A (2.40 Å), 1W58 (2.50Å) and 1W59 (2.70 Å) (Oliva et al. 2004) from *Methanococcus jannaschii*; 1RLU (2.08 Å), 1RQ2 (1.86 Å), 1RQ7 (2.60 Å) (Leung et al. 2004), 2Q1X (2.35 Å) and 2Q1Y (2.30 Å) (Respicio et al. database record) from *M. tuberculosis*; 1OFU (2.10 Å) (Cordell et al., 2003) and 2VAW (2.90 Å) (Oliva et al. 2007) from *Pseudomonas aeruginosa*; 1W5F (2.00 Å) (Oliva et al. 2004) from *Thermotoga maritima*. Most of these species belong to different phyla and subkingdoms of the *Bacteria* kingdom, and one, *M. jannaschii*, to the phylum *Euryarchaeota* (subkingdom *Archaea*). At the same time, there are a number of defects in all deposited in the Protein Data Bank structures. (Höltje et al. 2008) Most of deposited in PDB X-ray structures of bacterial FtsZs characterized by the loss of N- and C-terminal fragments (typically a few tens of residues), presence of gaps in protein globule, as well as absence of certain heavy atoms of side chains of amino acid residues.

Unfortunately, until now, there are no more or less complete X-ray structures of *E. coli* FtsZ protein, model organism also plays an important role in modern biological engineering and industrial microbiology. Currently only the 1F47 (PDB) structure have the last 17 amino acid residues (Lys367-Asp383), forming a short unstructured region, ends with a two-helix turn at the C-terminal end (Mosyak et al. 2000).

However, we are also interested in complete structure of this protein, due to the fact that commercial analytical kits for *in vitro* binding experiments are more available for *E. coli* FtsZ protein analysis than analytical kits for *M. tuberculosis*. *In vitro* and *in silico* modelling of interaction with low-molecular compounds of both, *E. coli* and *M. tuberculosis* FtsZ proteins, such as benzimidazole derivatives, allow us much accurate binding-site identification and analysis. Based on FtsZs structural homology, these experimentally confirmed binding site (or sites), can be extrapolated from *E. coli* protein to the structure of mycobacterial homolog. This allow us more accurate prediction of binding sites of such new and promising anti-TB compounds as benzimidazoles.

Thus, the purpose of the research was *in silico* modelling of three-dimensional structure of *E. coli* FtsZ protein, and qualitative reconstruction of *M. tuberculosis* FtsZ protein model based on comprehensive analysis of X-ray structures deposited in the Protein Data Bank.

Methods

Analysis of Protein Data Bank structures of FtsZ M. tuberculosis

Complete amino acid sequence of *M. tuberculosis* FtsZ (P64170) (Cole et al. 1998, Fleischmann et al. 2002) was downloaded from UniProt (http://www.uniprot.org/) database (The UniProt Consortium 2008). Multiple alignments of amino acid sequences of *M. tuberculosis* FtsZ PDB-structures and P64170 were realized in ClustalX 2.0.5 with a set of BLOSSUM matrices (http://www.clustal.org, Larkin et al. 2007). The PDB-structures of FtsZ *M. tuberculosis* protein were analyzed using »DeepView – Swiss-PdbViewer 4.0.3« (Guex and Peitsch 1997; http://www.expasy.org/spdbv/). In the absence of heavy atoms in the side-chains the program generated a warning notice about the type and location of structural defects. Lack of amino acid residues was detected by using Accelrys Discovery Studio Visualizer 3.0 (Accelrys Software Inc. – http://accelrys.com/).

Reconstruction of 3D model of E. coli FtsZ protein

Complete amino acid sequence of *E. coli* O157:H7 FtsZ (P0A9A8**)** (Perna et al. 2001) was downloaded from UniProt (http://www.uniprot.org/) database (The UniProt Consortium 2008). A three-dimensional structural modelling was carried out on the I-TASSER server (Roy et al. 2010; http://zhanglab.ccmb.med.umich.edu/I-TASSER) and with »SwissModel Automatic Modelling Mode« of »SWISS-MODEL Workspace« server (http://swissmodel.expasy.org/) (Arnold et al. 2006). Both servers running in automatic mode of PDB structure (template) selection. As a result we generated 5 models of three-dimensional structures with I-TASSER, and and one more model with »SWISS-MODEL Workspace«. Additionally, with »SWISS-MODEL Workspace« server we constructed another model based on 2Q1Y (Chain A) template PDB structure which was specified in the manual mode.

Root mean square deviations (RMSD) of the fitted 3-D structures were calculated using »molecule align« tool of PyMol 1.4 package (www.pymol.org).

Estimation of protein model quality

The 3-D structures quality was assessed by processing the models on the MolProbity server. (Chen et al. 2010, http://molprobity.biochem.duke.edu/) This study was performed to estimate the statistics of all-atom contacts (i.e. »all atoms Clashscore«) and protein geometry: defined percentages of poor rotamers, Ramachandran outliers, Ramachandran favoured, residues with bad bonds, residues with bad angles and defined Cβ deviations >0.25Å and MolProbity score.

We used the Protein Structure and Model Assessment Tools available at »SWISS-MODEL Workspace« server (http://swissmodel.expasy.org) to assess the quality of the 3-D models based on Raw-score and Z-score of QMEAN6 (Composite scoring function for model quality estimation) (Benkert et al. 2009) and global model quality estimation based on »DFire energy« (all-atom distance-dependent statistical potential) (Zhou and Zhou 2002).

Results

Selection and quality checking of X-ray Protein Data Bank structures of M. tuberculosis FtsZ

Scanning the Worldwide Protein Data Bank (wwPDB) we revealed several crystal structures of the *M. tuberculosis* cell division protein FtsZ, determined at 1.86 to 2,60 Å by X-ray method. The following PDB structures have been studied: 1RLU (Chains: A, B) and 2Q1Y (Chains: A, B) X-rays of FtsZ-GTPgammaS (5'-guanosine diphosphate monothiophosphate) complexes (Chains: A, B), 1RQ2 (Chains: A, B) and 2Q1X (Chains: A, B) X-rays of FtsZ-citrate complexes (Chains: A, B) and 1RQ7 X-ray of FtsZ-GDP complex (Chains: A, B). Using pairwise sequence alignment of polypeptide chains A and B from X-ray PDB structures with complete sequences from UniProt we tested them on presence of gaps (meaning occurrence of defects in structures). PDB-structures were verified on the presence of such artifacts as deficiency of heavy atoms (carbon, oxygen and nitrogen) in side chains of individual amino acid residues (using DeepView-Swiss-PdbViewer 4.0.3 software package). All gaps in polypeptide

chains and residues with defective side chains were checked and represented in the Figure 1 and Table 1.

As a result, of the ten available X-ray structures (considering chains A and B) the only one chain of *M. tuberculosis* FtsZ protein was selected as a most complete experimentally proved structure and the base of subsequent work on its detailed 3-D reconstruction and *in silico* analysis. Such structure was a chain A of X-ray FtsZ-GTP-gamma-S complex from 2Q1Y (2.30 Å, R-value=0.174, R-free=0.210) (DOI:10.2210/pdb2q1y/pdb). It has no just first 7 (N-end) and the last 66 (C-end) amino acid residues, but, as opposed to the same A-chain of 1RLU (2.08 Å, R-value=0.182, R-free= 0.224) (Leung et al., 2004), has a complete atomic composition of all available amino acids. The 2Q1Y chain A were analyzed with MolProbity server, and the values of evaluation functions (see Table 2), demonstrate its high quality for further *in silico* experiments and modelling its interactions with low molecular weight compounds.

Table 1: Features and identified defects in Protein Data Bank X-rey structures of *Mycobacterium tuberculosis* FtsZ.
Tabela 1: Lastnosti in okvare struktur PDB v proteinu FTsZ iz bakterije *Mycobacterium tuberculosis*.

PDB Strucrure	Method	Resolution, Å	Chain	Defective regions of molecules		
				N-terminal tile	Tubulin/FtsZ family, GTPase domain	C-terminal tail
1RLU	X-Ray Diffraction	2.35 Å	A	aa:M1-Y7	ha: E29, K33, R64, L66, R181	aa:D313 – R379; ha: K236, E252
			B	aa:M1-H5	aa:R60-G70,G170-A173; ha: N6, L8, E73, K77, K120, R140	aa:D313 – R379; ha: K236, D301
1RQ2	X-Ray Diffraction	1.86 Å	A	aa:M1-Y7	aa:R64-A69; ha: K33, M177	aa:D313 – R379; ha; K236
			B	aa:M1-Y7	aa:R60-G70, D171-A173; ha: K33, Q45, E73	aa:V314-R379
1RQ7	X-Ray Diffraction	2.60 Å	A	aa:M1-Y7	aa R64-A69; ha: K33, L48	aa:D313 -R379; ha: K236, Q255
			B	aa:M1-H5	aa:R60-G70, D171-A173; ha: K33, E73, K120, R140, S141, E153	aa:V314-R379; ha: K236, D313
2Q1X	X-Ray Diffraction	2.35 Å	A	aa:M1-Y7	aa:T63-G70	aa:D313 – R379
			B	aa:M1-H5	aa:R60-A71, R140-N142,Q168-A173	aa:D313 – R379
2Q1Y	X-Ray Diffraction	2.30 Å	A	aa:M1-Y7	-	aa:D313 – R379
			B	aa:M1-H5	aa:R60-G70, G170-A173	aa:D313 – R379

aa – lack of respective amino acid residues in the X-rey structure;
ha – absence of heavy atoms (carbon, oxygen or nitrogen) in the side chains of respective amino acid residues.

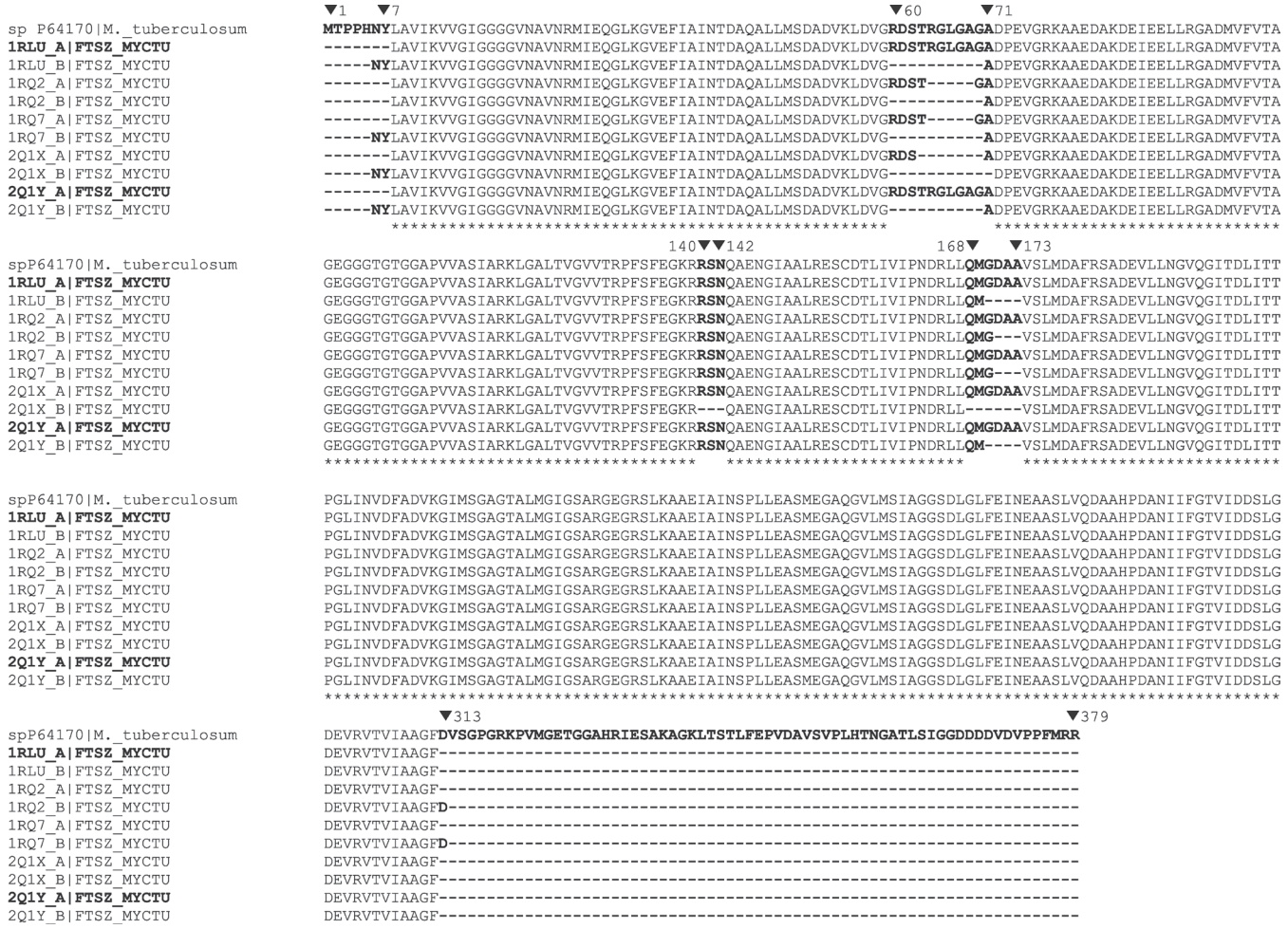

Figure 1: Numerous alignment of the full-length sequence of FtsZ protein (UniProtKB entry: P64170) from *Mycobacterium tuberculosis* and sequences of corresponding X-Ray Protein Data Bank structures: 1RLU, 1RQ2, 1RQ7, 2Q1X and 2Q1Y.

Slika1: Različice sekvence proteina FtsZ (UniProtKB (P64170)) iz bakterije *Mycobacterium tuberculosis* in sekvence pripadajočih struktur proteinov FtsZ z X žarki: 1RLU, 1RQ2, 1RQ7, 2Q1X and 2Q1Y.

E. coli FtsZ protein spatial structure prediction

Despite the great interest in mycobacterial FtsZ as the target for antibacterial compounds, majority of commercial analytical kits for *in vitro* binding experiments are more available for FtsZ protein from *E. coli* than its mycobacterial homolog. So, here we have a paradox situation, the presence of well proven three-dimensional structure of *M. tuberculosis* FtsZ protein on the one hand, and at the other hand, the fact that majority of the experimental tools targeted *E. coli* FtsZ, for which there is a clear gap in 3D-structure research. So, now, we have only 1F47 PDB structure presented only by last 17 amino acid residues (Lys367-Asp383), forming a short unstructured element, ending with two α-helix turns in C-end (Mosyak et al. 2000). In order to solve this problem we applied *in silico* homology modelling.

Initially the sequence of *E. coli* FtsZ protein (UniProt: P0A9A6) has been sent to the »SWISS-MODEL Workspace« server, for model building (alignment). With completely automatic modelling of *E. coli* FtsZ protein, server selected the chain B of the 1OFU X-ray structure from the SulA-FtsZ complex (2.10 Å, R-value=0.216, R-free=0.255) from *P. aeruginosa* (Cordell et al., 2003) as the template structure. »SWISS-MODEL Workspace« server generated one model for target FtsZ protein of 293 aminoacid residues in length (from Asn24 to Gly316 inclusive). The secondary and tertiary structures of model was completely similar to those in 1OFU except absence of small area in the N-end, covering the first β-fold and significant part of the next α-helix (Fig. 3a). In the structure of the matrix protein these elements are present (Ala11-Gly23 in FtsZ protein from *E. coli*, and Ala12-Ggly24 in FtsZ protein from *P. aeruginosa*). When the chain A of 2Q1Y structure was assigned as a matrix (previously selected as the best X-ray structure of *M. tuberculosis* FtsZ protein, see above), »SWISS-MODEL Workspace« server built model of *E. coli* FtsZ protein, which contained in its structure above-mentioned region (Fig. 2b) and was 305 amino acid residues in length (from Ala11 to Ile315 inclusive). Sequences of these structural areas in *E. coli* and *M. tuberculosis* FtsZ proteins have differences in amino acid residues: Ile16-Val14, Val18-Ile16 and G23-V20, respectively. Fitting of these two *E. coli* FtsZ structures, based on different modelling matrixes (Fig. 2c), demonstrate high level of structural similarity confirmed by root-mean-square deviation of Cα-atoms (RMSD=0.862 Å) (Höltje et al. 2008).

E. coli FtsZ protein spatial structure modelling performed by using I-TASSER server

In parallel with using of classical template-based modelling, we have applied 3D-reconstruction using on-line I-TASSER server (http://zhanglab.ccmb.med.umich.edu). I-TASSER 3D-models are built based on multiple-threading alignments by LOMETS (Wu and Zhang 2007) and iterative TASSER assembly simulations; function insights are then derived by matching the predicted models with protein function databases. (Roy et al. 2010) Following the sequence-to-structure-to-function paradigm, the I-TASSER procedure (Roy et al. 2010) for structure and function modelling involves four consecutive steps of: (a) template identification by LOMETS (Wu and Zhang 2007); (b) fragment structure reassembly by replica-exchange Monte Carlo simulations (Zhang et al. 2002); (c) atomic level structure refinement using REMO (Li et al. 2009) and FG-MD (Zhang et al. 2011); and (d) structure-based function interpretations using COFACTOR (Roy et al. 2011).

We submitted request of *E. coli* FtsZ-protein sequence (UniProt: P0A9A6) at the I-TASSER server using on default settings without manual assignment of template structure. As seen from Table 3, the server align our query sequence with a range of such template PDB structures as 2VAW (Chain A), 1FSZ (Chain A), 2VAM (Chain A), 2R6R (Chain A), 1W5F (Chain A) and 2RHL (Chain B). As a result, were generated five I-TASSER models of *E. coli* FtsZ protein of full length (from Met1 to Asp383) with different values of C-score (Table 4, Fig. 3). Model 1 has the highest value of C-score, indicating it as optimal model structure among generated by I-TASSER server. Based on server statistics Model 1 was also characterize by TM-score (template modelling score) = 0.56±0.15 and root-mean-square deviation (RMSD) = 9.6±4.6Å.

On the Figure 4 we present results of I-TASSER secondary structure and properties prediction for *E. coli* FtsZ protein. Confidence score values for the predicted structures are also indicating a

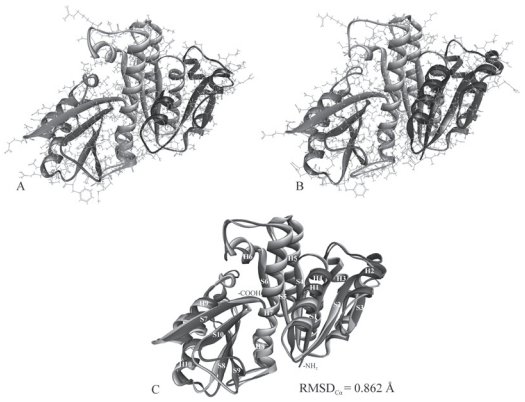

Figure 2: Spatial structure models of FtsZ protein from *Escherichia coli*, built with the protein structure homology-modeling server »SWISS-MODEL Workspace« using X-rey template PDB structures of different origin: A – FtsZ from *E. coli* constructed on template 1OFU (Chain B) from *P. aeruginosa;* B – FtsZ from *E. coli* constructed on template 2Q1Y (Chain A) from *M. tuberculosis*, C – Fitting of constructed 3-D models of FtsZ protein from *E. coli* (light gray – based on 1OFU (Chain B) template, dark gray – based on 2Q1Y (Chain A) template). Protein models presented as solid ribbon diagram with side-chain atoms shown as lines. 3-D models were visualized in Accelrys Discovery Studio Visualizer.

Slika 2: Tri dimenzionalni model FtsZ iz bakterije *Escherichia coli* narejen na serverju »SWISS-MODEL Workspace« ob uporabi matric različnih izvorov: A – FtsZ iz *E. coli* narejen na matrici 1OFU_B (vir – P. aeruginosa), B – FtsZ iz *E. coli* narejen na matrici 2Q1Y_A (vir – M. tuberculosis), C – Prileganje modela FtsZ narejenega iz *Escherichia coli* (svetlo sivo – matrica 1OFU_B, temno siva – matrica 2Q1Y_A). Model proteina predstavljen kot polni trak s stranskimi verigami atomov (linije). Izdelan v Discovery Studio Visualizer.

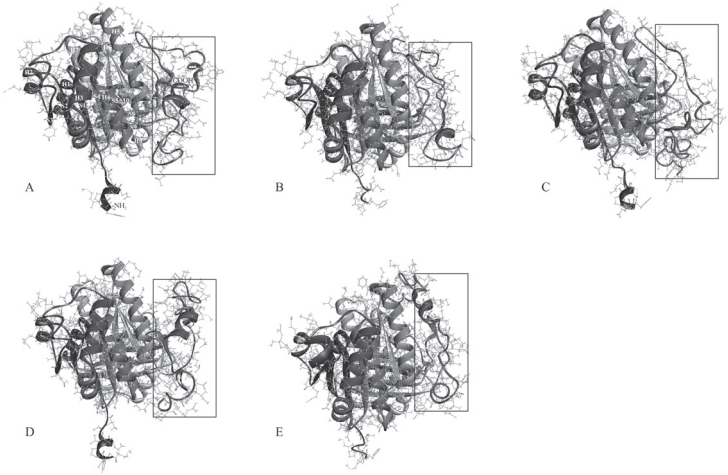

Figure 3: Spatial models of FtsZ *Escherichia coli* built on I-TASSER server.
A – model 1, B – model 2, C – model 3, D – model 4 and E – model 5. Protein models presented as solid ribbon diagram with side-chain atoms shown as lines. Boxes mark the C-terminal region that is absent in all crystal structures of FtsZ proteins and was overbuilt by the server based on sequence similarity to the regions of other classes proteins. Generated in Discovery Studio Visualizer.

Slika 3: Prostorski model FtsZ iz *Escherichia coli* narejen na I-TASSER serverju:
A – model 1, B – model 2, C – model 3, D – model 4 in E – model 5. Modeli proteinov so predstavljeni kot polni trak s stranskimi verigami atomov(linije). Okvir označuje C terminalno regijo, ki je odsotna v vseh strukturah kristala proteina FtsZ in je dograjena na serverju na osnovni podobnosti zaporedja v regijah pri ostalih proteinih. Izdelan v Discovery Studio Visualizer.

rather high quality of all built models. All this along with a variety of conformational representations of C-terminal region provided by five server-built models (Fig. 4) can not regard any from these structures as the quite probable and suitable for further bioinformatic research.

Quality evaluation of E. coli FtsZ protein models

All seven models of *E. coli* FtsZ were examined using the online MolProbity server and »Protein Structure & Model Assessment Tools« of »SWISS-MODEL Workspace« server.

Table 2: Results of evaluation of structure quality of chain 2Q1Y_A FtsZ *Mycobacterium tuberculosis* on a MolProbity server.
Tabela 2: Rezultati vrednotenja kakovosti strukture verige 2Q1Y_A FtsZ iz bakterije *Mycobacterium tuberculosis* na serverju MolProbity.

All-AtomContacts	Clashscore for all atoms:	4.13	96th percentile*
ProteinGeometry	Poor rotamers	0.00%	Goal: < 1%
	Ramachandran outliers	0.00%	Goal: < 0.2%
	Ramachandran favored	100.00%	Goal: > 98%
	Cβ deviations > 0.25 Å	0	Goal: 0
	MolProbity score	1.20	99th percentile*
	Residues with bad bonds:	0.00%	Goal: 0%
	Residues with bad angles:	0.00%	Goal: < 0.1%

Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.
* 100th percentile is the best among structures of comparable resolution; 0th percentile is the worst.
^ MolProbity score is defined as the following: 0.42574*log(1+clashscore) + 0.32996*log(1+max(0,pctRotOut-1)) + 0.24979*log(1+max(0,100-pctRamaFavored-2)) + 0.5

Table 3: Top 10 template X-Ray structures selected by I-TASSER server for homology modelling of *Escherichia coli* FtsZ protein
Tabela 3: Zgornjih 10 matric struktur z X žarki uporabljenih na I-TASSER-ju za modeliranje homolognosti FtsZ proteina bakterije *Escherichia coli*.

Rank	PDB Hit	Ident1	Ident2	Cov.	Norm. Z-score
1	2VAW (Chain A)	0.67	0.55	0.82	3.46
2	2VAW (Chain A)	0.67	0.55	0.82	5.88
3	1FSZ (Chain A)	0.44	0.40	0.86	6.38
4	2VAM (Chain A)	0.54	0.43	0.79	4.68
5	2R6R (Chain A)	0.46	0.39	0.84	6.20
6	2VAW (Chain A)	0.67	0.55	0.82	0.00
7	2VAW (Chain A)	0.67	0.55	0.82	13.50
8	1FSZ (Chain A)	0.48	0.26	0.53	5.77
9	1W5F (Chain A)	0.46	0.38	0.81	6.87
10	2RHL (Chain A)	0.52	0.43	0.82	5.00

(a) Rank of templates represents the top ten threading templates used by I-TASSER.
(b) Ident1 is the percentage sequence identity of the templates in the threading aligned region with the query sequence.
(c) Ident2 is the percentage sequence identity of the whole template chains with query sequence.
(d) Cov. represents the coverage of the threading alignment and is equal to the number of aligned residues divided by the length of query protein.
(e) Norm. Z-score is the normalized Z-score of the threading alignments. Alignment with a Normalized Z-score >1 mean a good alignment and vice versa.

Table 4: MolProbity server and »Protein Structure and Model Assessment Tools« of »SWISSMODEL Workspace« server structure quality evaluation for 3-D models of *Escherichia coli* FtsZ protein.

Tabela 4: Rezultati vrednotenja kakovosti strukture 3-D modela FtsZ iz bakterije *Escherichia coli* na serverju MolProbity in »Protein Structure and Model Assessment Tools« na serverju »SWISS-MODEL Workspace«.

Verification Type		Models from I-Tasser *C-score*					Template based models from Swiss-Model		Goal
		Model 1-*1.239*	**Model 2**-*1.726*	**Model 3**-*2.056*	**Model 4**-*2.638*	**Model 5**-*2.396*	1OFU_B	2Q1Y_A	
All-Atom contacts	Clashscore, all atoms:	121.27(0th percentile*)	177.41(0th percentile*)	122.68(0th percentile*)	141.22(0th percentile*)	131.33(0th percentile*)	38.41(9th percentile*)	48.84(4th percentile*)	Less is better
Protein geometry	Poor rotamers	1.36%	2.38%	2.38%	3.06%	2.72%	1.83%	0.00%	<1%
	Ramachandran outliers	3.15%	2.89%	3.41%	3.94%	1.84%	0.34%	0.33%	<0.2%
	Ramachandran favored	92.39%	89.76%	89.50%	89.76%	92.65%	95.53%	98.68%	>98%
	Cβ deviations >0.25Å	9	13	5	9	3	2	0	0
	MolProbity score^	3.12(19th percentile*)	3.55(8th percentile*)	3.40(11th percentile*)	3.54(8th percentile*)	3.37 (11th percentile*)	2.58(43rd percentile*)	2.16(67th percentile*)	Less is better
	Residues with bad bonds:	0.00%	0.52%	0.00%	0.00%	0.00%	0.00%	0.00%	0%
	Residues with bad angles:	5.74%	6.27%	5.74%	4.18%	4.70%	0.00%	0.00%	<0.1%
DFire energy		–494.67	–455.72	–471.66	–461.74	–471.66	–377.70	–412.32	Less is better
QMEAN Raw score 6		0.614	0.62	0.628	0.595	0.592	0.684	0.747	Higher value is better
QMEAN Z-score 6		–1.79	–1.72	–1.62	–-2.01	–2.04	–0.96	–0.31	Less negative is better

Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.

* 100th percentile is the best among structures of comparable resolution; 0th percentile is the worst.

^ MolProbity score is defined as the following: 0.42574*log(1+clashscore) + 0.32996*log(1+max(0,pctRotOut-1)) + 0.24979*log(1+max(0,100-pctRamaFavored-2)) + 0.5.

DFire is an all-atom statistical potential based on a distance-scaled finite ideal-gas reference state. It's used to assess non-bonded atomic interactions in the protein model. A lower energy indicates that a model is closer to the native conformation.

QMEAN6 scoring function is a linear combination of six structural descriptors using statistical potentials: The local geometry is analysed by a torsion angle potential over three consecutive amino acids. Two distance-dependent interaction potentials are used to assess long-range interactions: the first is a residue-level implementation based on C-beta atoms only and the second an all-atom potential which is able to capture more details of the model. A solvation potential investigates the burial status of the residues. Two additional terms describing the agreement of the predicted (from sequence) and the calculated secondary structure and solvent accessibility of the model.

```
                          20                  40                  60                  80                  100
                          |                   |                   |                   |                   |
Sequence        MFEPMELTNDAVIKVIGVGGGGGNAVEHMVRERIEGVEFFAVNTDAQALRKTAVGQTIQIGSGITKGLGAGANPEVGRNAADEDRDALRAALEGADMVFIAAGMGGGTGTG
Prediction      CCCCCCCCCCCSSSSSSSCCCHHHHHHHHHCCCCCSSSSSSSCHHHHHCCCCCCSSSCCCCCCCCCCCCCCHHHHHHHHHHHHHHHHHHCCCSSSSSCCCCCCCCCC
Conf.Score      98743367897399997578528999999983998648999825199996399981797054124668898838889999975999999977899899935878887666
Solv.Acces.     72434437740301000001000000120154605201000000001004416062204014400410101132620450044126303500630200000010111211 1

                      120                 140                 160                 180                 200                 220
                       |                   |                   |                   |                   |                   |
Sequence        AAPVVAEVAKDLGILTVAVVTKPFNFEGKKRMAFAEQGITELSKHVDSLITIPNDKLLKVLGRGISLLDAFGAANDVLKGAVQGIAELITRPGLMNVDFADVRTVMSEMGY
Prediction      HHHHHHHHHHCCCCSSSSSCCCCCCCCHHHHHHHHHHHHHHHHHCCSSSSSCCHHHHHCCCCCCHHHHHHHHHHHHHHHHCHHHHHCCCCCCCCCCHHHHHHHHCCCC
Conf.Score      68999999998299789998378463678999999999999997698899945688975379998999999999999987089998647883025679999999668 83
Solv.Acces.     00100030046130000000012152614302430450053037201000000142015016771303300430140033002100100322010000012021104432 2

                          240                 260                 280                 300                 320
                           |                   |                   |                   |                   |
Sequence        AMMGSGVASGEDRAEEAAEMAISSPLLEDIDLSGARGVLVNITAGFDLRLDEFETVGNTIRAFASDNATVVIGTSLDPDMNDELRVTVVATGIGMDKRPEITLVTNKQVQQ
Prediction      SSSSSSSSCCCCHHHHHHHHHHCCCCCCCCCCCCCSSSSSSSCCCCCCHHHHHHHHHHHHHHHCCCCCSSSSSSSCCCCCCSSSSSSSSSCCCCCCCCCCCCCCCCCC
Conf.Score      66787786484199999999971946578885787259999935999878999999999999749997099864688877981899999816886655666666766556 6
Solv.Acces.     00001030326510340043014111024130630310000000033020310330042036102660100000000460643020000000124645464543645435 4

                      340                 360                 380
                       |                   |                   |
Sequence        PVMDRYQQHGMAPLTQEQKPVAKVVNDNAPQTAKEPDYLDIPAFLRKQAD
Prediction      CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCHHHHHCCC
Conf.Score      65554445567876545677776677676667787656500788754 69
Solv.Acces.     44454454454444445644445445544444465442020031036348 1
```

Figure 4: Results of secondary structure and properties prediction for I-TASSER model 1 of *Escherichia coli* FtsZ protein
Secondary structure elements are shown as »H« for α-helix, »S« for β-sheet and »C« for coil. Conf.Score is confidence score values (higher values for better). Values range for predicted solvent accessibility (Solv.Acces.) vary from 0 (buried residue) to 9 (highly exposed residue). Bold and underlined are selected a sequences of FtsZ protein from *E. coli* models buildet on »SWISS-MODEL Workspace« server and based on 1OFU (Chain B) and 2Q1Y (Chain A) templates respectively.

Slika 4: Napovedana sekundarna struktura in značilnosti prvega modela FtsZ iz bakterije *Escherichia coli* na serverju I-TASSER.
Sekundarni strukturni elementi so prikazani kot as »H« za alfa vijačnico, »S« za beta list in »C« za zanko. »Conf.Score« je merilo zaupanja (višje vrednosti pomeni bolje).Območje vrednosti za napovedno dostopnost topila (Solv.Acces.) poteka od 0 (tesno vezan ostanek) do 9 (visoko bremenjen ostanek). Odebeljeno in podčrtano so izbrana zaporedja modela FtsZ E. coli model narejenega na serverju »SWISS-MODEL Workspace« na osnovi matric 1OFU_B in 2Q1Y_A.

Evaluation of all functions demonstrates a significantly higher quality of models, built with SWISS-MODEL server (Table 4). Evaluation of all-atom contacts, namely the number of serious steric overlaps of all atoms, allows us to determine model based on PDB matrix structure 1OFU (Chain B) as the best among all *E. coli* FtsZ protein models. At the same time, evaluation indexes of protein geometry, listed in Table 4, characterize another SWISS-MODEL structure, based on PDB template 2Q1Y (Chain A), as the most accurate.

According to all-atom statistical potential DFire index, among I-TASSER models, the undisputed leader are the Model 1 and an outsider – Model 4. At the same time, among SWISS-MODEL reconstructed models, structure, based on 2Q1Y (Chain A), demonstrate substantially superior quality in comparison with model based on *Pseudomonas* FtsZ structure. Evaluation indices »Raw score« and »Z-score« of QMEAN6, demonstrate undeniable superiority of *E. coli* FtsZ model based on 2Q1Y (Chain A) template.

Discussion

Choice of FtsZ M. tuberculosis model

As shown in Figure 1, polypeptide chains of all FtsZ structures deposited in the database are lacking the first 5-7 a.a. residues of N-terminal region and 65-66 residues of C-terminal region (in fact, the last C-terminal region is absent at all). Also, eight chains, which are representing all five of the studied PDB-structures of FtsZ *M. tuberculosis* (in 1RLU and 2Q1Y – only Chains B), contain 5-10 residues gap within the loop, which corresponds to the loop between sheet S3 and helix H3 from 1FSZ – crystal structure of FtsZ protein from *M. jannaschii* (Oliva et al. 2004). B-chains of each structure are characterized by the absence of 3-6 residues in the loop that extend to helix H7. Additionally, the chain B of 2Q1X has a short gap (3 a.a. residues) within the α-helix that corresponds to helix H5 of 1FSZ. Relative to the artifacts of residues, only 2Q1X and 2Q1Y crystals have all heavy atoms in all amino acid residues of structure (Table 1). Both of this two structures of FtsZ *M. tuberculosis* are characterized by complete composition of amino acid side chains, whereas the X-Rey structure obtained by Leung et al. 2004 (PDB: 1RQ7) are suffer from lack of some heavy atoms in at least three residues in each chain.

Thus, Chain A from the 2Q1Y structure, which does not possess the seven initial (N-end) and sixty six C-end amino acid residues but in contrast to the Chain A from 1RLU has full atom composition of all available amino acid residues, in our opinion is a good three-dimensional model of FtsZ *M. tuberculosis*. This assumption was confirmed by the scores of all MolProbity quality evaluating functions for this structure (model) (Table 2).

Construction and verification of FtsZ E. coli models

The »SWISS-MODEL Workspace« is a web-based integrated service dedicated to protein structure homology modelling. It assists in building protein homology models at different levels of complexity. Successful model building requires at least one experimentally determined 3D structure (template) that shows significant amino acid sequence similarity with the target sequence. Building a homology model comprises four main steps: identification of structural template(s), alignment of target sequence and template structure(s), model building, and model quality evaluation. These steps can be repeated until a satisfying modelling result is achieved. Each of the four steps requires specialized software and access to up-to-date protein sequence and structure databases. In full automatic mode server performs all four steps itself and gives the complete three-dimensional model of the target protein with specified matrix structure. There is also a possibility to specify the necessary structure as the matrix manualy (Arnold et al. 2006).

Thus, in full automatic modelling of FtsZ *E. coli* server used Chain B from 1OFU structure as a matrix. It should be noted that selection of this object as a matrix was based on the origin of these two bacterial species. Both of them are belonging to the class γ-proteobacteria from phylum Proteobacteria. Although orders of these species are different, an affiliation to a common class is important enough to explain/suppose a higher level of similarity between sequence of

FtsZ from *E. coli* and *P. aeruginosa* (Vaughan et al. 2004, Demchuk and Blume 2005), than with sequences from other bacterial species from different phylums for which crystal structures are also presented in PDB database.

However, such relatively close relationship between *E. coli* and *P. aeruginosa* was not sufficient to build a complete model of FtsZ *E. coli*. Incomplete N-terminal end of this model starting with the Asn24, which indicates the absence of first beta-sheet and significant part of the next alpha-helix in the model structure (Fig. 2a), although in the structure of the matrix this elements are present (Ala11-Gly23 in FtsZ from *E.coli* and Ala12-Gly24 in FtsZ from *P. aeruginosa*). This artifact is surprising because of the fact that primary sequence of missing part of FtsZ *E. coli* is completely similar to that area in the template structure – FtsZ protein from *P. aeruginosa.*

We can only assume some failure in the algorithm of »SWISS-MODEL Workspace« server taking note the fact that the using a chain 2Q1Y_A as specific template allowed server to generate FtsZ *E. coli* model, which contains the mentioned area in its structure (Fig. 2b) and that is why it was stretched from the Ala11 to Ile315 inclusive.

Cα-atom RMSD of obtained FtsZ *E. coli* models, whics were built on different matrices (Fig. 2c), is 0.862 Å. This indicates a significant structural similarity of the obtained models (Gu and Bourne 2009). And it can become an additional demonstration of the structure conservatism of this class of proteins (Erickson, 1998) and significant modelling accuracy while taking proteins from bacteria phylums class as a template. Minor differences observed in the turn of main chain (Fig. 2c) are confined to the unstructured elements between ß-sheets and alpha-helices and fully capable of leveling through the lability of the secondary structure elements in the time (Nyporko and Blume 2001).

In parallel with the modelling on »SWISS-MODEL Workspace« server we have performed modelling of FtsZ *E. coli* on I-TASSER server, an Internet service for protein structure and function predictions. It built 3-D models based on multiple-threading alignments by LOMETS and iterative TASSER assembly simulations; function insights are then derived by matching the predicted models with protein function databases. I-TASSER (as 'Zhang-Server') was ranked as the No 1 server for protein structure prediction in recent CASP7, CASP8 and CASP9 experiments (http://predictioncenter.org/). It was also ranked as the best for function prediction in CASP9. CASP (or Critical Assessment of Techniques for Protein Structure Prediction) is a community-wide experiment for testing the state-of-the-art of protein structure predictions which takes place every two years since 1994. The experiment (often referred as a competition) is strictly blind because the structures of testing proteins are unknown to the predictors (Roy et al. 2010, http://zhanglab.ccmb.med.umich.edu/I-TASSER/about.html).

As a result, server generated five potential full length models of FtsZ proteins from *E. coli* (Fig. 3) with different values of C-score (see Table 4). C-scores a confidence score for estimating the quality of predicted models by I-TASSER. It is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. C-score is typically in the range of [-5,2], where a C-score of higher value signifies a model with a high confidence and vice-versa (Roy et al. 2010). As it is shown in the Table 4 the best value of this parameter belongs to the model 1.

I-TASSER server estimated accuracy for this model: 0.56±0.15 TM-score and 9.6±4.6 Å RMSD. TM-score and RMSD are known standards of structural similarity between two structures which are usually used as measure of model accuracy when the native structure is known. In case where the native structure is not known, it becomes necessary to predict the quality of the modelling prediction, i.e. what is the distance between the predicted model and the native structures. TM-score is a recently proposed scale for measuring the structural similarity between two structures (Zhang and Skolnick 2004). The purpose of proposing TM-score is to solve the problem of RMSD which is sensitive to the local error. Because RMSD is an average distance of all residue pairs in two structures, a local error (e.g. a misorientation of the tail) may cause a significant RMSD value although the global topology may be correct. In TM-score, however, the small distance is weighted stronger than the big distance which makes the score insensitive to the local modelling error. A TM-score >0.5 indicates a model of

correct topology and a TM-score <0.17 means a random similarity. This cutoff does not depend on the protein length (Roy et al. 2010). Thus, the quality of prediction of model 1 is quite satisfactory for these scores.

On Fig. 3 we presented a predicted secondary structures and properties of FtsZ protein from *E. coli* models generated by I-TASSER. It should be noted that the distribution of secondary structure elements (shown as H for alpha helixes, S for beta sheets and C for coils) matches with that for crystal structures of FtsZ (see figure 2 in Oliva et al., 2007). Confidence score values for the proposed structure also indicate a rather high quality of all predicted models. However, it should be noted that the value of this estimated parameter are at a slightly lower level for the C-tail region (which is not represented in any of the crystal structures of FtsZ proteins) in comparison with the globular N- and C-domains. We can speculate the possibility of predicted by I-TASSER server unstructured conformation of short N-terminal region in the structure of FtsZ *E. coli* because analogs are observed in structures 2VAW and 1FSZ. But predicted secondary structure of long and not structured C-terminal region between B10-sheet and final α-helix, provided in the FtsZ *E. coli* (Mosyak et al. 2000), does not inspire confidence in us.

Low values of Confidence score and some crystal structures of FtsZ testified against coiled coil structure of this C-terminal region provided by I-TASSER server. Thus, in contrast to several crystal FtsZ structures that are starting with ß-sheet B1 and breaking immediately after the ß-sheet B10 (1OFU, 1RQ7, 1RLU, 1RQ2, 2Q1X and 2Q1Y), FtsZ structures from 2R6R and 2R75 as well as 2VAP and 1FSZ have two additional ß-sheets B11 and B12. 1W5X structure has also a short α-helix between B10 and B11, which is terminated element of C-end region in structures 1W5F, 2RHH and 2RHJ. All together with a variety of conformational representations of this C-terminal region in five models provided by I-TASSER server (Fig. 4) can not regard any of these models as the most likely and suitable for further bioinformatics researches.

For the final determination of the best potential model of FtsZ *E. coli* among all constructed we have verified all seven candidates with the online server MolProbity and »Protein Structure & Model Assessment Tools« of »SWISS-MODEL Workspace«.

MolProbity allows to evaluate the quality both of all atoms contact and so the protein geometry of any three-dimensional biopolymer molecule. Evaluation of all-atom contacts, specifically the number of serious steric overlaps of all atoms, allows to choose model, built basing on template 1OFU_B, as a favourable. In this case second model from »SWISS-MODEL Workspace« loses much less for this parameter in compare to models of I-TASSER. On the other hand, estimation of all parameters of protein geometry (Table 4), allow us to consider the »SWISS-MODEL Workspace« model built based on template structure 2Q1Y (chain A), as the best one. However, on such parameters as »poor rotamers« and »Ramachandran favoured« it's not much better than the second model from this server and all models from I-TASSER. A significant benefit of both models from »SWISS-MODEL Workspace« over models from I-TASSER is observed in rates of »Ramachandran outliers«, »Cβ deviations«, »MolProbity score«. Significant differences are also observed in »Residues with bad angles« parameter. While two models from »SWISS-MODEL Workspace« have no residues with bad angles, in structures from I-TASSER models the quantity of these residues reaches 4-6 %. It should be noted that residues with bad bonds observed only in the case of model 2 among all seven analyzed models.

Benefits of models built on the I-TASSER in all-atom statistical potential »DFire energy« due to the bigger size of this models that are completely full unlike to the incomplete models from SWISS-MODEL server. Therefore, this parameter from »Protein structure & model assessment tools« we used to compare structures built only on the same server.

Finally, for evaluating quality of three-dimensional structures, it was implemented QMEAN6 score (http://swissmodel.expasy.org/qmean/cgi/index.cgi). QMEAN6 is a reliability score for the whole model which can be used in order to compare and rank alternative models of the same target. The quality estimate ranges between 0 and 1 with higher values for better models. Additionally, the pseudo energies of the four contributing statistical potential terms are provided as well as the percentage agreement

between predicted and measured features from the sequence and model, respectively. The comparison of the differences of the terms among the models may help to understand the reason for the differences in the estimated model quality. In addition to the »Raw scores«, »Z-scores« of the QMEAN composite score as well as all terms are provided relating the quality estimates to scores obtained for high-resolution reference structures solved experimentally by X-ray crystallography (Benkert et al. 2011). The QMEAN »Z-score« represents an measure of the absolute quality of a model by providing an estimate of the »degree of nativeness« of the structural features observed in a model and by describing the likelihood that a given model is of comparable quality to experimental structures. Models of low quality are expected to have strongly negative QMEAN Z-scores (i.e. the model's QMEAN score is several standard deviations lower than expected for experimental structures of similar size). Evaluation QMEAN6 scores, especially »Raw score« and »Z-score«, puts everything in its place, clearly demonstrating the undeniable advantage of *E. coli* FtsZ protein model based on template 2Q1Y (chain A) over all other constructed models.

Among the variety of crystallographic structures of FtsZ proteins from tuberculous pathogen *M. tuberculosis*, deposited in »Protein Data Bank«, we have chosen chain 2Q1Y (chain A) as a reasonable model for further bioinformatics researches. It lacks first seven and last 66 residues, but it is characterized by full atom composition of all available residues and excellent estimated values of parameters from MolProbity.

Also, among the seven potential FtsZ *E. coli* models, built by I-TASSER server and »SWISS-MODEL Workspace«, we have selected a model from the last server, based on matrix 2Q1Y (chain A). This model prevailed for qualitative evaluation parameters not only full atom models of I-TASSER, but model, built on a matrix 1OFU (chain B), which represents FtsZ structure from *P. aeruginosa* – bacterial species systematically much closer to *E. coli* (the same class of γ-proteobacteria), than *M. tuberculosis*.

Thus, were have successfuly reconstructed 3-D models of the FtsZ proteins of *E. coli* and *M. tuberculosis* is of sufficient quality for further *in silico* studies such as molecular docking, molecular dymamics simulations and computational drug discovery.

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The role of cell selection for pollen grain fertility after treatment of barley sprouts (*Hordeum distichum* L.) with UV-B irradiation

Pomen izbora celic za plodnost pelodnih zrn po obravnavanju kalic ječmena (*Hordeum distichum* L.) z UV-B sevanjem

Elena Kravets

Department of Genomics and Molecular Biotechnology, Institute of Food Biotechnology and Genomics, National Academy of Sciences of Ukraine, 2a Osipovskogo Str., Kyiv 04123, Ukraine
correspondence: kravetshelen@gmail.com

Abstract: UV-B irradiation of barley sprouts within the range of 0.5-4.3 kJ/m2 induced an increase in the number of chromosome aberrations in the root meristem and pathologies in the reproductive system. Enhancement of cytomixis, increase of polymorphism and cytopathology of pollen grains were observed in the male generative system. The inverse trend was observed when intensity of cytomixis was compared to the pollen grain sterility. Damages induced by low doses of UV-B radiation were eliminated neither by DNA reparation nor by cell selection and were preserved in many cell generations. High UV-B level led to the activation of cytomixis due to which the population of microsporocytes was released from the excess load. It is presumed that cytomixis present a form of cell selection which was induced by an excess of microsporocyte disturbances.

Keywords: chromosome aberrations, root meristem, microsporangium, cytomixis, pollen grain sterility, cell selection, UV-B radiation, *Hordeum distichum* L.

Izvleček: Obravnavanje kalic ječmena z UV-B sevanjem (od 0,5-4,3 kJ/m^2) je povzročilo nastanek številnih kromosomskih aberacij v rastnem meristemu korenin in motnje v reproduktivnem sistemu. Pri moških generativnih organih smo zasledili povečanje citomikse, polimorfizma in patoloških sprememb pelodnih zrn. Ugotovili smo negativno povezanost med jakostjo citomikse ter pogostostjo patoloških sprememb pri tetradah mikrospor in sterilnostjo pelodnih zrn. Poškodbe zaradi nizkih odmerkov UV-B sevanja s popravljanjem DNA in z izborom celic niso izginile in so se ohranile več generacij celic. Visok odmerek je povzročil citomikso, zaradi česar se je sprostila populacija mikrosporocit. Predvidevamo, da citomiksa predstavlja način izbora celic, ki nastane zaradi večjega obsega motenj mikrosporocit.

Ključne besede: kromosomske aberacije, koreniski meristem, mikrosporangij, citomiksa, sterilnost peloda, izbor celic, UV-B sevanje, *Hordeum distichum* L.

Introduction

Ultraviolet in the range of 280-320 nm affect all levels of plant organization and also signal, regulatory and energetic functions (Jordan 1996, Ziska et al. 1992, Ziska and Teramura 1992, Caldwell et al. 2003, 2007; Zhang et al. 2003, Koti et al. *2004 a, b,* 2007*,* Hectors et al. 2010, Ballaré et al. 2011, Krasylenko et al. 2011). One of the most significant effects of enhanced UV-B radiation is an injury of the reproductive function of plants. It has been shown that additional UV-B radiation can exert genotoxic effect on the meristem, inhibit growth and development, influence pollination, decrease the quantity of the produced pollen and seed production of plants (Flint and Caldwell 1984, Conner and Neumeier 2002, Koti et al. 2004 a, b, 2007). Besides the direct action on generative organs, the main target of which is DNA of cells, UV-B radiation produces indirect effects which are realized by mechanisms connected with photoreception, transduction of signals and hormonal regulation (Tevini et al. 1981, Flint and Caldwell 1984, Santos et al. *1998;* Caldwell et al., 2007, Demkura et al. 2010, Keller et al. 2011*)*. The effect of action depends in many cases on genotype, ecotype, the stage of ontogenesis and other reasons (Jordan 1996, Torabinejad et al. 1998, Caldwell et al. 2007, Li et al. 2010). The data on mechanisms of the effect of UV-B radiation on the generative organs of plants are not available. In this connection, the main goal of the paper was to elucidate the character of damages in the generative system induced by UV-B radiation, its dose dependence, and also to estimate the role of cell selection in normalization of the pollen grain fertility.

Material and methods

We used barley (*Hordeum distichum* L., 2n=14) of Scarlet variety of French selection. Three-day sprouts were irradiated by a 20 W Philips TL ultraviolet lamp with filter cutting off the short-wave region of the ultraviolet spectrum. Radiation doses were 0.5, 2.2 and 4.3 kJ/m^2 with the intensity 0.5 W/m^{2}s^1. One group of sprouts (about 100 plants) was fixed 24 and 48 hour after irradiation. The other group of sprouts (about 100 plants) was cultivated in soil to study the development of reproductive organs. The material was fixed with Navashin mixture; temporal slides were prepared according to the standard cytological protocol (Pausheva 1984). The fixation of spike was made from the differentiation stage of microsporocytes to maturation of pollen grains. The slides were stained with acetoorcein following enzymic maceration (for root meristem) and either acetocarmin or Schiff's reagent (Feulgen protocol) for microsporangium. The quantity of anomalies in cell systems was counted and measurements were made.

The calculation of the number of chromosomal aberrations was carried out by the ana- telophase method, the mitotic index (MI) was defined by percentage of mitotic dividing cells. We estimated 10 roots per stage, 50-70 anthers with microsporocytes and 20 anthers for the analysis of pollen grains (PG) per stage. The data were statistically processed by the Microsoft Excel software.

Results

The effects of UV-B-radiation on the root meristem.

In the first mitosis the quantity of chromosome aberrations increased in proportion to the radiation dose, in the second mitosis the dose dependence varied: at high dose the level of chromosome aberrations decreased, while the number of degenerated cells increased (Table 1, Fig. 1). The cell degeneration occurred by the apoptosis type. It was particularly remarkable that in individual cases under maximal exposition of UV we observed the phenomena of cytomixis connected with migration of the injured chromatin along the plasmodesmal channels. Therefore, the increasing of UV-B-radiation dose resulted in increased number of aberrations in the first mitosis, while in the second, the induction dynamics of chromosome aberrations exhibited both direct and inverse dose-dependence (Fig. 1).

The effects of UV-B radiation on the reproductive system of plants.

Microsporogenesis: Microsporogenesis was with the formation of tetrads of the isobilateral

N/N		Root meristem				Microsporogenesis and the stage of the development of the pollen grain				
Experimental variant.		24 h after irradiation		48 h after irradiation		Microsporogenesis, degree of cytomixis, %	Tetrads with anomalies, %	Sterility of microspores, B %	Sterility of two-celled PG, %	Sterility of the three-celled PG, %
Radiation dose		MI, %	Aberrant anaphases, %	MI, %	Aberrant anaphases, %					
1	Control	5.8	3.05±0.65	4.5	2.20±0.29	4.3	4.5±0.3	1.9±0.1	3.8±0.9	2.9±0.7
2	0.5 kJ/m^2	6.2	2.61±0.73	5.2	5.75±0.96	7.3	6.0±0.6	2.1±0.3	9.0±1.7	11.3±1.4
3	2.2 kJ/m^2	6.6	5.92±0.96	5.1	6.24±0.80	6.3	8.1±1.0	2.7±0.4	9.7±1.5	9.1±1.8
4	4.3 kJ/m^2	5.8	8.82±1.10	5.0	4.52±0.82	19.7	8.5±0.7	3.7±0.4	6.8±1.5	3.8±0.8

Table1: The indices of cytogenetic disturbances in the root meristem of *H. distichum* sprouts and the generative sphere of the plants in the process of ontogenesis.
Tabela 1: Indeksi citogenetskih motenj v meristemu korenin kalic vrste *H. distichum* in generativnih organih v procesu ontogeneze.

structure is presented in Figures 3a and 4a. Under the influence of UV-B-radiation, cytomixis was the main type of pathology in microsporogenesis. We consider that one should distinguish between weak (local), intensive and destructive (pathological) cytomixis. Local cytomixis is a physiological norm for barley. UV-B radiation, similar to other stress factors, intensifies the destructive character of cytomixis. In barley under maximal exposition of UV intensive cytomixis affected up to about 20% of microsporocytes (Fig. 3b). In this case stickenessand fluidity of chromatin increased in microsporocytes. We observed a kind of transitional chromatin (fragments of nuclei, chromosomes, micronuclei, bands of chromatin from cell to cell). Not all microsporangia were affected by cytomixis. Most of microsporocytes completed meiosis with the formation of normal, only rarely nonbalanced, tetrads of microspores (Figs. 4a, b). In barley the bulk of »transitional« chromatin usually remained either in the composition of cynticia or in the intracellular space. Destructive cytomixis usually occurred in less developed flowers and in immature spikes of the second growth and is, presumably, a way to eliminate the nonviable cell system.

The dose dependence of cytomixis incorporation into microsporogenesis was of nonlinear character (Fig. 1). In this case, low correlation was observed between the intensity of cytomixis and frequency of pathologies in tetrads of microspores. Though cytomixis might be the reason for formation of the unbalanced tetrads, both types of disturbances were, most likely, a result of the same reason associated with genetic instability (prolonged mutagenesis) induced by the effect of UV-B- radiation.

The development of pollen grain: Under normal conditions, the development of male gametophyte in barley, similar to most of cereals, begins to release microspores from the microsporocyte envelope and includes the stages completing the formation of sporoderm, growth and polarization of microspore. Then follows the first asymmetric mitosis, polarization of two-celled pollen grain, the second mitotic division which are accompanied by the synthesis of cytoplasm, and then by the deposition of reserve substances in the vegetative cell cytoplasm (Batygina 1974, Poddubnaya-Arnoldi 1976, Heslop-Harrison 1979, Mascarenhas 1989).

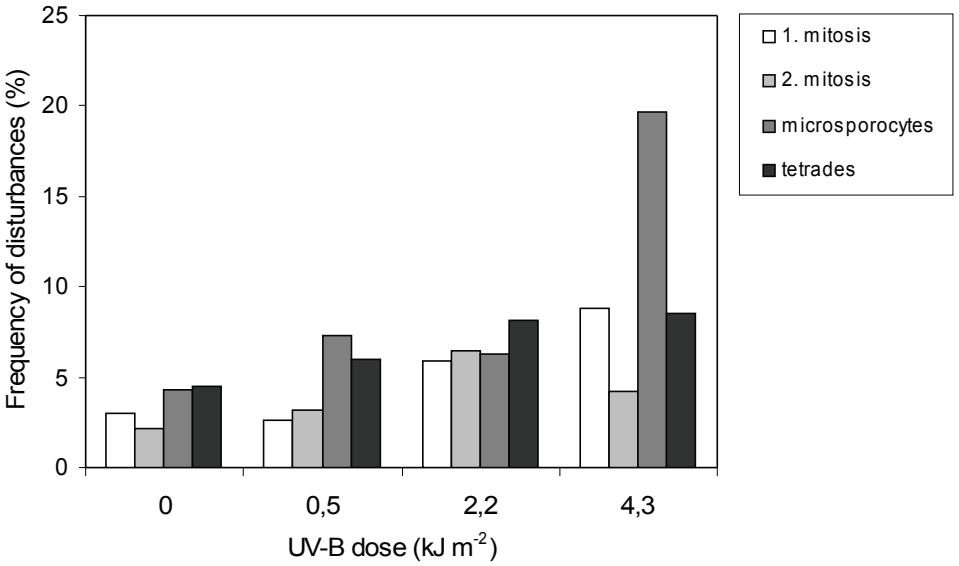

Figure 1: UV-B dose dependences of the number of cytogenetic disturbances in *H. distichum* sprouts in the first mitosis in the root meristem and microsporogenesis.
Slika 1: Število citogenetskih motenj v odvisnosti od UV-B sevanja pri kalicah *H. distichum* pri prvi mitozi v koreninskem meristemu in mikrosporogenezi.

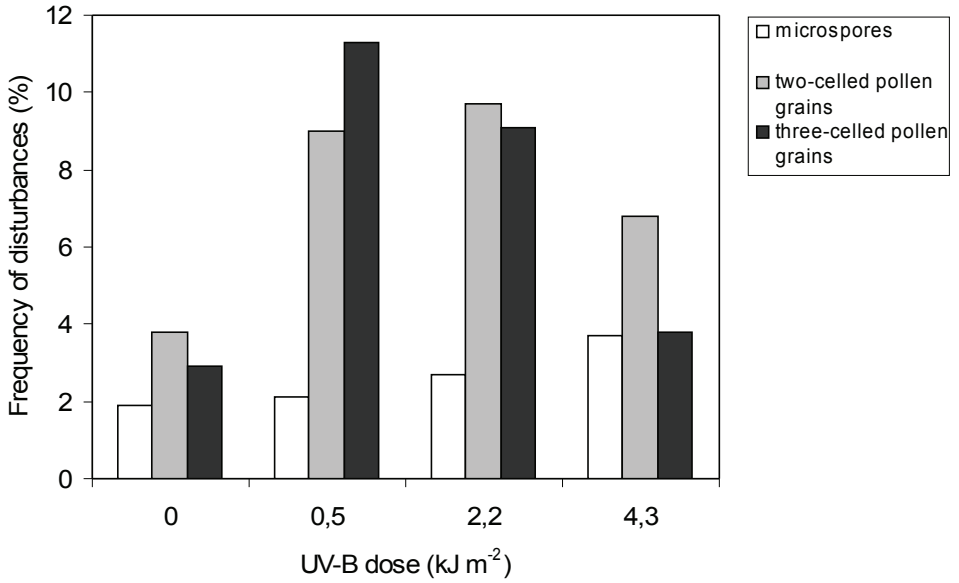

Figure 2: UV-B dose dependences of the number of cytogenetic disturbances of *H. distichum* in the phases of development of pollen grain.
Slika 2: Število citogenetskih motenj pri vrsti *H. distichum* v fazah razvoja pelodnega zrna.

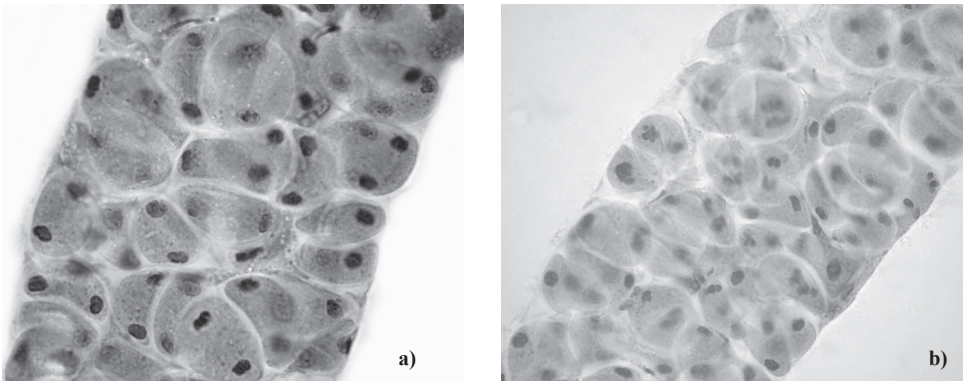

Figure 3: Microsporogenesis: a – telophase of 2nd divisions of meiosis, control; b – telophase of 2nd divisions of meiosis, UV-B dose: 4.3 kJ\m^2; intensive cytomixis.
Slika 3: Mikrosporogeneza: a – telofaza druge delitve pri mejozi, kontrola; b – telofaza druge delitve pri mejozi, UV-B odmerek: 4,3 kJ\m^2, intenzivna citomiksa.

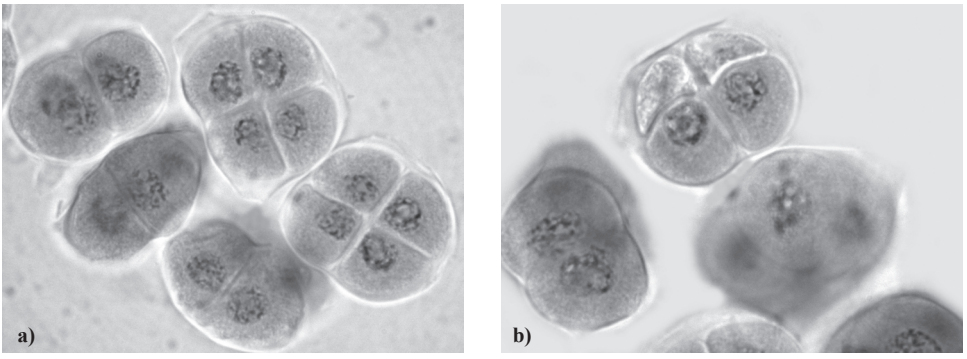

Figure 4: Formation of the tetrads: a – UV-B dose: 0.5 kJ\m^2; b – UV-B dose: 4.3 kJ\m^2.
Slika 4: Nastanek tetrad: a – UV-B odmerek: 0,5 kJ\m^2; b – UV-B odmerek: 4,3 kJ\m^2.

The mature pollen grain of barley has a pair of arrow-shaped sperms and a vegetative cell nucleus, the cytoplasm of which is filled with amyloplasts. Ultraviolet-B radiation led to an enhancement of polymorphism and to disturbance of polarity in pollen grains, unsynchronized development, the increase of the frequency in the formation of oligoplasm pollen grains (Fig. 5a). The latter present an evidence for nonspecific character of gametic disturbances caused by different stress factors. The appearance of oligoplasm pollen grains might be associated by either mutations of specific genes of pollen grain, whose expression intensifies after the first mitosis or by mutation which determines the male cytoplasmic sterility (Mascarenhas 1990, Nirmala and Kaul 1994). Such pollen grains are late or interrupt in their development, and their sperms do not complete the cycle of their differentiation. According to the morphological traits degeneration of microspore nucleus, generative cell, sperms and nucleus of the vegetative cell in the pollen grain occur by apoptosis (Fig. 5b). In reality, the recent publication reports that UV-B irradiation can indeed initiate apoptotic processes in plant cells (Lytvyn et al. 2010).

Thus, the range of cytological disturbances in pollen sacs had a nonspecific character.

Induction of disturbances in the course of pollen grain development correlated negatively with the UV-B radiation dose and with the degree

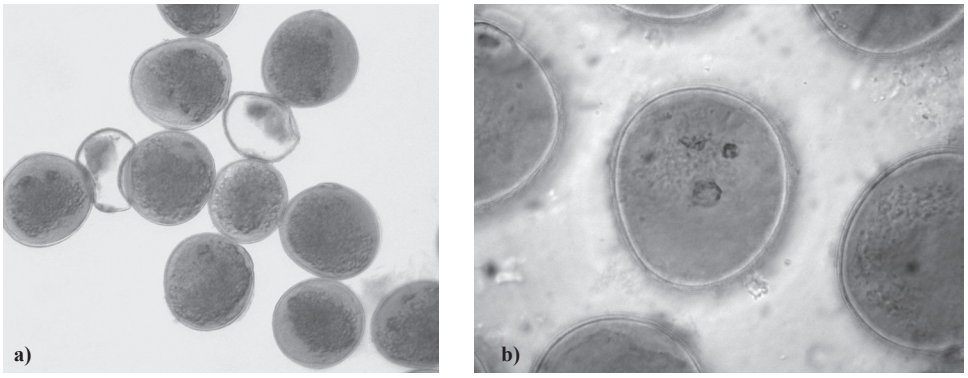

Figure 5: Mature three-celled pollen grains: a – normal and »oligoplasm« pollen grains, UV-B dose: 2.2 kJ\m^2; b – apoptosis degradation of pollen grain nuclei, UV-B dose: 4.3 kJ\m^2.
Slika 5: Zrela tri-celična pelodna zrna: a – normalna in »oligoplazemska« zrna, UV-B odmerek: 2,2 kJ\m^2; b – apoptozni razpad jedra pri pelodnemu zrnu, UV-B odmerek: 4,3 kJ\m^2.

of cytomixis (Table 1, Fig. 2). An enhancement of the UV-B dose in first step increased the number of anomalous pollen grains, but further on it decreased. At the higher dose the level of pollen sterility was close to control.

Discussion

This might evidenced the threshold character of the effect and the induction of the recovery mechanisms. It is known that in response to increased level of cytogenetic disturbances the DNA reparation systems become more active and leading to apoptosis induction or to proliferative death of non-repaired cells via the cell selection (Calendo 2001). Due to cell selection, namely, to the haplontic cell selection (taking place at the ontogenesis haplophase) the cells with recessive lethal mutations that are not subjected to the action of diplontic cell selection are eliminated (Gaul 1959). The action of haplontic selection is evident at the stage of the pollen grain maturation.

Paradoxically, but disturbances induced by low doses of UV-B radiation were not eliminated neither by reparation nor by cell selection and are preserved in many cell generations. They remained beyond the reach even of the haplontic competition resulting in relatively high percentage of the pollen grain sterility. Cytomixis began to act at higher UV-B-radiation dose, due to which the population of microsporocytes released from the excess of genetic load. Consequently, in the response of plants to radiation the threshold effect was observed which may be caused by damages of a number of DNA and other molecules initiating the reparation processes and cell selection (Calendo 2001). The intensification of cell selection occured in ontogenesis at the end of diplophase (microsporogenesis) and also at the end of haplophase (formation of gametes). Thus the long-term effects of UV radiation on reproductive system are likely causing the prolonged mutagenesis which was induced by irradiation. Decrease of disturbance number in reproductive tissues at the higher dose UV is probably connected with the threshold effect and activation of restore processes.

Cytomixis as a form of cell selection, occurring in microsporocytes before and at the beginning of meiosis, deserves special attention. We presumed that *via* cytomixis the population of microsporocytes regulated its excess, and simultaneously eliminated from the mutational load and solved the problems of nutritional character. Space continuity and uniqueness of microsporocytes as a cenocytic system of pollen sac were realized via cytomixis (Heslop-Harrison 1966a, 1966b, Welan 1974, Guo and Zheng 2004). Cytomixis might play a special role in provision of repairing processes in initials of male gametes.

In reality in the case of local cytomixis the chromatin loops penetrate the intercellular chan-

nels and united microsporocytes into groups in the early prophase of meiosis. Such contacts do not entail negative consequences for future meiosis. *Vice versa*, the microsporocytes that are not included in the network may fall out of the developmental program. They may be delayed in the prophase-metaphase of the first division of meiosis and undergo proliferative death. It is known the callose wall of microsporocytes is not an impermeable barrier and does not prevent the migration of chromatin and organelles topenetrate through wide intercellular channels along which not only chromatin but also cytoplasmic organelles, signal molecules and trophic factors may pass (Risueno et al. 1969, Zheng et al. 1987, Souza and Pagliarini 1997, Hecht 2000). It is believed that the intercellular contacts ensure not only the synchronization of meiosis but also the cellular population homogeneity in microsporocytes and equalize the qualitative state of pollen grains which is required for a rapid and successful pollination.

Cellular selection in the population of microsporocytes occurs through the so-called autonomous apoptosis which unlike morphogenetic apoptosis, is not programmed but is initiated by the microsporocyte population itself. This assumption is supported by the irregularity of cytomixis, which does not occur simultaneously in all microsporangia or microsporocytes in the same anther. Intensive cytomixis is induced by UV-B radiation, hybridization and other stress factors. Many researchers assume that the nature of intensive and destructive cytomixis is connected with the genetic disbalance (disturbance of homeostasis) of polyploids, haploids, aneuploids, mutants, hybrids and apomicts (Poddubnaya-Arnoldi 1976, Mantu and Sharma 1982, Bedi 1990, Orlova 1994). Stress factors, such as radiation, hybridization, chemical agents and herbicides usually enhance the destructive effect of cytomixis (Bobak and Herich 1978, Dwivedi et al. 1988, Bellucci et al. 2003). Usual environmental conditions and their seasonal fluctuations exert no marked effect on cytomixis, which is obviously under the genetic control (Mantu and Sharma 1982, Bellucci et al. 2003).

The intensive and destructive (to a larger extent) cytomixis made the meiosis pattern more complicated and may led to serious genetic consequences. However, this is mainly peculiar to genetically unbalanced, sterile forms and in spikes of the second growth or nonviable individuals. Basing in the experimental material and data from literature we assumed that cytomixis reflected the mechanisms of the cellular selection during which cellular population limits the number of functioning microsporocytes, thus regulating redundancy, and eliminating the mutation load.

Conclusion

UV-B radiation of barley sprouts within the range of 0.5-4.3 kJ/m^2 induced an increase in the number of chromosome aberrations in the root meristem and pathologies in the reproductive system. Enhancement of cytomixis, increased polymorphism and cytopathology of pollen grains was observed in the male generative system. Injuries caused by UV-radiation were of nonspecific character. The negative correlation was observed between the intensity of cytomixis and frequencies of pathologies in tetrads of microspores and also between the sterile level of pollen grains. The induction of disturbances during development of the pollen grain revealed negative dose dependence. Under the maximal exposition of UV-B radiation the index of pollen sterility approaches to that obtained at the control. Injuries induced by low doses of ultraviolet were not eliminated, either by DNA reparation either by cell selection and were preserved in many cell generations. An enhancement of the radiation dose led to the activation of cytomixis due to which the population of microsporocytes was released from the excess load. We presume that cytomixis present a form of cell selection which is induced by an excess of the injuries a threshold level of microsporocytes. It possibly limits mutagenesis, regulates the state, quantity of microsporocyte population solving simultaneously the problems of nutritional character and thus promotes preservation of the pollen grain fertility.

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Defence responses of Norway spruce seedlings to elicitors of ectomycorrhizal fungus *Pisolithus tinctorius* and pathogen *Heterobasidion annosum* are affected by zeatin riboside

Vpliv zeatin ribozida na obrambni odgovor kalic smreke po tretiranju z elicitorji mikorizne glive *Pisolithus tinctorius* in patogena *Heterobasidion annosum*

Matevž Likar, Marjana Regvar

Department of Biology, Biotechnical Faculty, University in Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia
*correspondence: matevz.likar@bf.uni-lj.si

Abstract: Cytokinins are known to attenuate defence responses of plants after elicitor application or inoculation with fungi. To evaluate their role in the regulation of colonisation of Norway spruce (*Picea abies*) seedlings with mycorrhizal and pathogenic fungus, we analysed the effects of zeatin riboside (ZR) on: i) growth of ectomycorrhizal fungus *Pisolithus tinctorius* and pathogen *Heterobasidion annosum* in axenic cultures, ii) colonisation intensity of selected fungi on *P. abies* seedlings and iii) induction of defence reactions of spruce seedlings following elicitor treatment. Mycorrhizal fungus *P. tinctorius* showed increased growth at concentrations higher than 10^{-2} µM ZR that was accompanied by increased ergosterol concentrations. In contrast, decreased growth of pathogen *H. annosum* was observed at the highest ZR (10 µM) concentration. ZR treatment also increased colonisation of spruce seedlings with the mycorrhizal fungus. Application of cell wall preparations of both fungi increased peroxidase (POD) activity in the roots of treated spruce seedlings, whereas only elicitors of *H. annosum* increased also phenylalanine ammonia-lyase (PAL) activity, levels of soluble phenolics and salicylic acis (SA) concentrations. Application of ZR negated the increased activity of POD that was observed in elicitor treated seedlings, accompanied by increased levels of soluble phenolics in the roots of elicitated seedlings. In contrast, no effects of ZR treatment on PAL activity and SA accumulation could be observed. Our results suggest involvement of ZR in the regulation of cell wall modifications during the fungal colonisation with *P. tinctorius* and formation of ectomycorrhizae, by affecting the growth of fungal partner and non-specific defence reactions of the plant host.

Keywords: cytokinins, peroxidases, phenolics, phenylalanine ammonia lyase, *Picea abies*

Izvleček: Iz literature je znano, da lahko citokinini vplivajo na obrambne reakcije rastlin, ki se sprožijo po aplikaciji elicitorjev ali ob inokulaciji z živo glivo. Za ovrednotenje njihove vloge pri regulaciji kolonizacije kalic smreke (*Picea abies*) z mikoriznimi in patogenimi glivami, smo preverili vpliv zeatin ribozida (ZR): i) na rast ektomikorizne glive *Pisolithus tintorius* in patogena *Heterobasidion annosum*

v aksenični kulturi, ii) na stopnjo kolonizacije kalic smreke z obema glivama in iii) na aktivacijo obrambnih reakcij smreke po tretiranju z elicitorji. V aksenični kulturi smo pri 10^{-2} µM koncentraciji ZR opazili pospešeno rast mikorizne glive *P. tinctorius*, ki jo je spremljala povečana koncentracija ergosterola v miceliju. Nasprotno je bila rast patogene glive *H. annosum* pri najvišji koncentraciji ZR v gojišču (10 µM) zavrta. Podobno kot v aksenični kulturi je dodaten ZR pospešil kolonizacijo kalic smreke z ektomikorizno glivo, medtem ko na stopnjo kolonizacije s patogenom ni imel učinka. Tretiranje smreke z elicitorji obeh gliv je povečalo aktivnost peroksidaz (POD) v koreninah kalic, samo elicitorji patogene glive *H. annosum* pa so povečali tudi aktivnost fenilalanin amonijeve liaze (PAL) in koncentracijo topnih fenolov ter proste salicilne kisline (SA). Dodatek ZR je znižal peroksidazno aktivnost v kalicah tretiranih z elicitorji obeh gliv in povečal koncentracijo topnih fenolov. Nasprotno, ZR ni imel nobenega vpliva na aktivnost PAL in akumulacijo salicilne kisline v koreninah smreke. Na podlagi naših rezultatov predvidevamo, da je ZR vpleten v regulacijo modifikacij celične stene ob glivni kolonizaciji z ektomikorizno glivo *P. tinctorius* in vzpostavitev ektomikorize, preko delovanja na glivnega partnerja in nespecifične obrambne reakcije gostitelja.

Ključne besede: citokinini, peroksidaze, fenil alanin amonijeva liaza, *Picea abies*

Introduction

Root exudate is a very diverse group of compounds released from the roots to the rhizosphere and contains along with amino acids, organic acids, proteins and sugars also several plant growth regulators, including cytokinins (Neumann and Römhled 2000). Cytokinins play a crucial role in regulating proliferation and differentiation of plant cells, and also control various processes in plant growth and development (Sakakibara 2006; Kyozuka 2007). Furthermore, cytokinins are known to influence the growth of fungi (Barker and Tagu 2000, Nasim and Rehman 2006).

Colonisation of plants by different symbiotic and pathogenic fungi triggers the expression of defence related genes and induces local and systemic host responses (Ryals et al. 1996; Hammerschmidt 1999). An almost ubiquitous feature of plant responses to fungal colonisation or elicitors treatment is the activation of phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) and peroxidases (EC 1.11.1.7), which are associated with phenolic chemistry and cell wall modifications (Chitoor et al. 1997). It was demonstrated that cytokinins can regulate activity and expression of peroxidases (Kadioglu and Durmus 1997, Limam et al. 1998) and can therefore affect the reactions leading to cell-wall reinforcements. When tobacco plants were wounded in the presence of the synthetic cytokinin, benzylaminopurine, production of jasmonic acid (JA) was accompanied by salicylic acid (SA) accumulation (Sano et al. 1996), which is normally not accumulated upon wounding (Pieterse and Van Loon 1999). These results indicated that cytokinins may be involved in accumulation of JA and SA (Sano et al. 1996), which transmits the defence activation signal from the point of infection throughout the plant (Yalpani et al. 1993). Though majority of information concerning the role of PAL, peroxidases and SA in plant defence emanates from research on angiosperms, several studies suggest a similar role in host responses of gymnosperm Norway spruce (Asiegbu et al. 1994, Kozlowski and Metraux 1998, Nagy et al. 2000, Nagy et al. 2004; Likar and Regvar 2008).

In the present study we tested the effects of zeatin riboside (ZR) on the growth of the ectomycorrhizal fungus *Pisolithus tinctorius* and necrotrophic pathogen *Heterobasidion annosum*, as differential effects of cytokinin treatment on growth and colonisation of host roots with both fungal groups (ectomycorrhizal vs. necrotroph) was assumed. Ergosterol content in the fungal mycelia was tested in combination with the fungal growth studies, due to its importance in the formation of sterol rich domains (Xu et al. 2001), which are involved in several important processes

such as endocytosis and hyphal growth (Alvarez et al. 2007). Further, the effects of ZR on the colonisation intensity and induction of defence reactions of spruce seedlings following elicitor treatment were examined, as cytokinins were already proven to affect activity and expression of some enzymes involved in the defence reactions (Kadioglu and Durmus 1997, Limam et al. 1998). Several differences in the spruce seedling responses after colonisation with *P. tinctorius* and *H. annosum* were observed previously (Likar and Regvar 2008) and are assumed to be at least in part influenced by cytokinins.

Material and methods

Effects of zeatin riboside on fungal growth

Cultures of ectomycorrhizal fungus *Pisolithus tinctorius* (Mich.: Pers.) Coker and Couch (isolate DB49) andet al. necrotroph *Heterobasidion annosum* (Fr) Bref. (isolate DB75) are maintained in the fungal collection of Plant Physiology Lab (Biotechnical Faculty, University of Ljubljana). Sequences of ITS-rDNA region of both fungal species can be obtained from GenBank under accession numbers EU559631 and EU559632. Fungal cultures were grown on Melin-Norkrans-Marx (MNM) media (Marx 1969), supplemented with zeatin riboside (ZR) at final concentrations: 0, 10^{-6}, 10^{-4}, 10^{-2}, 1 and 10 µM. Due to different growth rates, growth of the individual fungus was monitored for two weeks in case of *H. annosum* and five weeks in case of *P. tinctorius*, after which mycelia were removed and frozen in liquid nitrogen, prior to freeze-drying (Christ, Alpha 2-4).

Ergosterol analysis

Twenty mg of dried fungal material were used for ergosterol extraction following modified protocol of Martin et al. (1990). For ergosterol quantification, 25 µl of ethanolic supernatant was injected into the high performance liquid chromatography (HPLC) system consisting of Waters 2960 separation module with Waters 996 PDA detector. UV absorbing compounds were separated by reverse-phase chromatography on a 5-µm Spherisorb C18, 250 mm x 4.6 mm column. The mobile phase was 100% methanol at 1 ml min^{-1}. Ergosterol peak was identified by comparison of the retention time (absorbance at 280 nm) and absorbance spectra with the purified ergosterol standard (Nylund et al. 1992).

Elicitation and inoculation experiments

Seeds of Norway spruce (*P. abies* (L.) Karst. prov. Čermošnjice) were surface sterilised in 30% H_2O_2 (105 min), washed and sown on sterile peat: vermiculite (1:3, V/V) mixture supplemented with Knop's medium (Booth 1971). Seedlings were grown in growth chamber (16h of light, 23 °C) for three months, and then transferred to Petri dishes for the elicitation and colonisation experiments.

For the elicitor treatment, fungal cell wall extracts of both fungal species were prepared as described by Salzer et al. (1996). For elicitation of spruce seedlings, elicitors were added to the MNM media at final concentration of 0.1 g l^{-1} (Likar and Regvar, 2008). To half of the seedlings ZR was added to MNM media at final concentration of 10^{-3} µM (determined as optimal for spruce seedling growth prior to experiments), while sterile double distilled water was used for the control. After ten days of growth the seedlings were collected and whole roots excised. Roots were freeze-dried (Christ, Alpha 2–4) and grinded to powder using liquid nitrogen prior to extraction of proteins and soluble phenolics.

For the inoculation experiments three months old spruce seedlings were transferred to petri dishes and covered with semi-circles of cloth without (control) or with the fungi, according to Chilvers et al. (1986). An additional semicircle of cotton soaked with half strength liquid MNM media without sugars was laid over the roots. Inoculated seedlings were grown for one month, after which colonisation of the spruce seedlings was estimated under a stereomicroscope (Leica, MZ8) by counting the short roots that had been entered by the hyphae.

PAL and peroxidase activity

Soluble proteins were extracted in 100mM phosphate buffer (pH 7.0, 1:10 = w/V) as described

by Albrech et al. (1994). Supernatant was used for total protein quantification, as well as PAL and POD assays. A modified Lowry assay (Sandermann and Strominger 1972) with bovine serum albumin as the standard was used for quantification of total soluble proteins prior to enzyme assays. Concentrations of soluble proteins ranged from 1 to 2 mg ml^{-1}.

Peroxidase activity was measured as described by Bashan et al. (1987), using guaiacol as substrate in an assay mixture containing: 25 µl of the protein extract, 0.5 ml 100 mM phosphate buffer, 0.2 ml 1% guaiacol and 0.2 ml 10 mM H_20_2. The enzyme activity was measured by monitoring the increase in absorbance at 470 nm (extinction coefficient of 26.6 mM^{-1} cm^{-1}) during the polymerization of guaiacol into tetraguaiacol (Chance and Maehly 1955).

PAL was assayed spectrophotometrically following a modified method of Khan and Vaidyanathan (1986). The PAL assay was performed at 37 °C for 1 h in an assay mixture containing: 100 µl of the protein extract 450 µl 50 mM TRIS-HCl (pH 8.8) and 25 µl 100 mM L-phenylalanine. The PAL activity was measured by monitoring the increase in absorbance at 290 nm (extinction coefficient of 16.6 mM^{-1} cm^{-1}) during the production of t-cinnamic acid (Gomez-Vasquez et al. 2004).

Total soluble phenolics

Soluble phenolics in the roots of spruce seedlings were extracted subsequently in 90% methanol and 100% methanol (1:25, w/V). Total phenols in combined methanolic extracts were measured according to Marigó (1973). For this, 1 ml of 2% Na_2CO_3 and 75 µl of Folin-Ciocalteau reagent (Kemika, Zagreb) were added to 100 µl of phenolic extract. After 15 minutes of incubation at 25 °C in the dark, the absorbance at 750 nm was measured (HP 8452A spectrophotometer). Catechin (Sigma) was used as a standard.

Analysis of salicylic acid (SA)

SA was extracted following the modified procedure of Raskin et al. (1989). The initial extraction procedure was the same as for the extraction of soluble phenolics. Pooled methanol extracts were dried and resuspended in 0.5 ml 5% (w/V) trichloroacetic acid. After 1 min of mixing by sonification (35kHz, Elma, Transonic 460/H), the extracts were centrifuged for 10 min at 3,000 x *g* (4 °C), and the supernatants were extracted twice with 0.5 ml of ethylacetate: cyclopentane (1:1, V/V). The organic phases were pooled and dried at 40 °C. The dried extracts were resuspended in 1.5 ml 100% methanol and filtered (0.22 µm) before injecting onto the HPLC.

For the analysis of SA, we used the same HPLC system configuration as above with additional flourimetric 474 detector. SA was identified and quantified by monitoring fluorescence at 407 nm (the excitation wavelength was 305 nm). The elution was carried out using: A – 20 mM sodium acetate buffer (pH 5.0) with 0.02% sodium azide (Sigma); and B – 100% methanol (Merck). The gradient used was: 0–4.5 min: 75% A; 4.5–11 min: 75%-30% A; 11-15 min: 30% A, at a flow rate of 1 ml min^{-1}, with 100 µl of sample injected per run.

Statistical analysis

Experiments were repeated three times. As similar trends were observed in all experiments represented data is from a single experiment.

Effects of elicitor and ZR treatment were determined by analysis of variance according to general linear model procedure. Differences among various treatment means were separated by Holm-Sidak post hoc test, while effects of ZR treatment on colonisation were evaluated using t-test. All analyses were performed at the 0.05 level of probability in SigmaStat (SPSS).

Results

Growth and ergosterol content of fungi in axenic cultures

ZR treatment increased colony diameter and ergosterol concentration of mycorrhizal fungus *P. tinctorius* at concentrations higher than 10^{-2} µM ZR (Fig 1). In contrast, pathogen *H. annosum* showed a decrease in colony diameter at the highest ZR (10 µM ZR) concentration. Ergosterol concentrations in the mycelia of *H. annosum* showed only

marginal effects of ZR, as we could observe an increase in the ergosterol concentration only at 10^{-2} µM concentration of ZR.

Fungal colonisation

One month after inoculation, colonisation of spruce seedlings with ectomycorrhizal fungus *P. tinctorius* reached 40.2% ±5.3%, while pathogen *H. annosum* colonised 58.9%±6.7% of the root tips. ZR treatment of spruce seedlings significantly increased (t-test, $p < 0.05$) the colonisation with mycorrhizal fungus *P. tinctorius* to 73.4% ±9.0% (a 82% increase in colonisation levels). No effects of ZR treatment on the colonisation of spruce seedlings with the pathogen *H. annosum* were observed (colonisation of ZR treated seedlings was 63.0%±6.9%).

Enzyme activity and phenolics

Only treatment with elicitors from *H. annosum* increased the PAL activity in the roots of the treated seedlings (Fig. 2a), whereas no effects of elicitors of *P. tinctorius* or ZR on the PAL activity could be observed.

Treatment with elicitors from both fungi induced POD activity in the roots of the treated seedlings (Fig. 2b). ZR treatment decreased POD activity in seedlings treated with elicitors from both fungi, whereas no effects of ZR on POD activity were observed in the control seedlings.

Treatment with elicitors of necrotroph *H. annosum* increased root levels of soluble phenolics (Fig. 2c), whereas no increase in accumulation could be observed in seedlings treated with elicitors of *P. tinctorius*. ZR increased the levels of soluble phenolics for 20% in the roots of the seedlings treated with elicitors of *H. annosum* and for 28% in the seedlings treated with elicitors of *P. tinctorius*, but had no effect on the levels of soluble phenolics in the control seedlings.

Treatment with elicitors of *H. annosum* increased also the root levels of free SA to 1.6 times the levels of the control (Fig. 2d), whereas elicitors of *P. tinctorius* had no effect on the root SA levels. Similarly to PAL activity, ZR treatment did not affect the SA concentrations in the elicitor treated nor control seedlings.

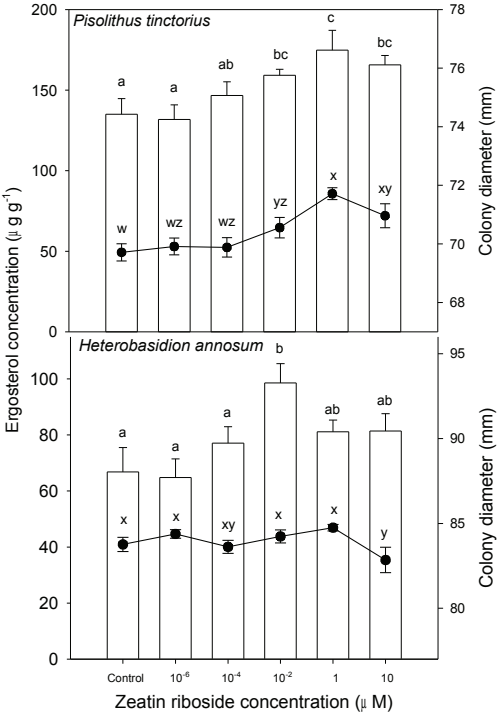

Figure 1: Ergosterol concentration (bars) and colony diameter (●) of *Pisolithus tinctorius* and *Heterobasidion annosum* treated with zeatin riboside at 10^{-6} to 10 µM concentration (Means±SE, n = 10 for colony diameter and n = 6 for ergosterol concentrations). Letters depict statistically significant difference of one-way ANOVA and Holm-Sidak post hoc test at $p < 0.05$.

Slika 1: Koncentracija ergosterola (stolpci) in premer kolonije (●) gliv *Pisolithus tinctorius* in *Heterobasidion annosum* rastočih na gojišču z 10^{-6} µM – 10 µM koncentracijo zeatin ribozida (SV±SN, n = 10 za premer kolonij in n = 6 za koncentracije ergosterola). Črke predstavljajo statistično značilno razliko testa enosmerne ANOVA in Holm-Sidak post hoc testa pri $p < 0,05$.

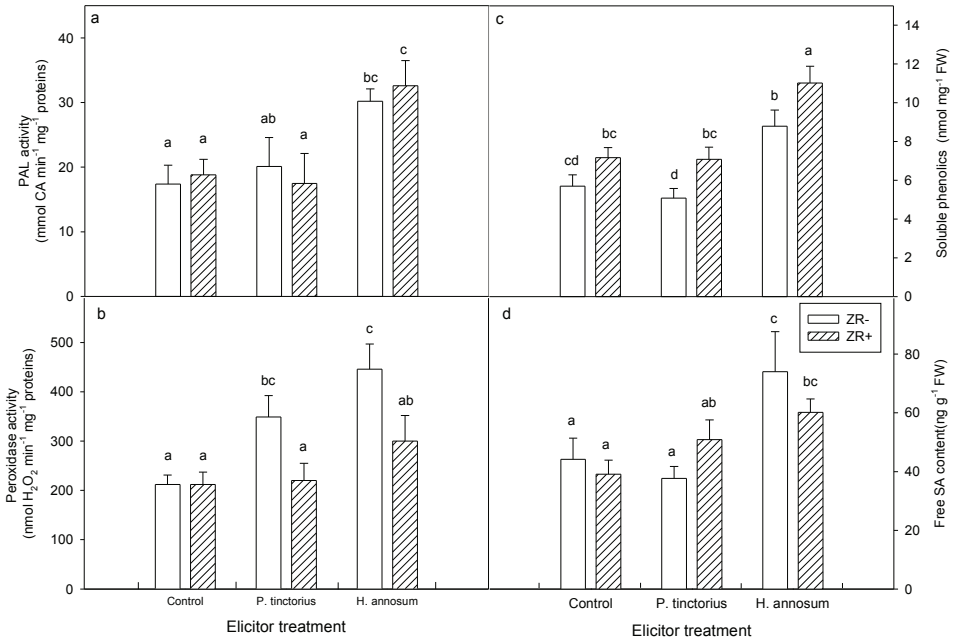

Figure 2: Impact of elicitors of *Pisolithus tinctorius* or *Heterobasidion annosum* and zeatin riboside treatment on: a) phenylalanine ammonia lyase activity, b) peroxidase activity, c) soluble phenolics and d) free salicylic acid concentrations in roots of the spruce seedlings (Mean±SE, n = 10). Letters depict statistically significant difference of one-way ANOVA and Holm-Sidak post hoc test at $p < 0.05$.

Slika 2: Vpliv tretiranja z elicitorji gliv *Pisolithus tinctorius* ali *Heterobasidion annosum* in zeatin ribozidom na: a) aktivnost fenilalanin amonijeve liaze, b) peroksidazna aktivnost, c) koncentracija topnih fenolov in d) koncentracija proste salicilne kisline v koreninah kalic smreke (SV±SN, n = 10). Črke predstavljajo statistično značilno razliko testa enosmerne ANOVA in Holm-Sidak post hoc testa pri $p < 0,05$.

Discussion

ZR treatment increased mycelial growth of ectomycorrhizal fungus *P. tinctorius* at concentrations, similar to the concentrations found in soil extracts (Van Staden and

Dimalla 1976) and *in vitro* cultures of mycorrhizal fungi (Wullschleger and

Reid 1990, Kovac and Žel 1995). Stimulating effects of cytokinins on growth of mycelia of ectomycorrhizal fungi were observed also by other authors (Gogala and Pohleven 1976, Pohleven 1988). Pohleven (1988) also observed increased fluidity of the plasma membranes of the hyphae of *Suillus variegatus* after cytokinin treatment, which is believed to contribute to increased growth of fungal mycelia. In our experiments ZR mediated increase in growth of *P. tinctorius* was accompanied by increased ergosterol concentrations. Ergosterol is an important constituent of the lipid rafts and sterol rich domains (SRD), which have been implicated in fungi in important processes such as endocytosis, virulence and hyphal growth (Alvarez et al. 2007; Steinberg et al. 2007). Due to the importance of ergosterol as a constituent of SRD and its high ability to promote the formation of SRDs (Xu et al. 2001), observed changes in ergosterol concentrations could be connected to morphological and physiological changes observed by Martin et al. (2001) and increased radial growth observed in our experiments. Positive effects on radial growth of ectomycorrhizal fungus *P. tinctorius* could support the importance of cytokinins in the regulation of the formation of the mycorrhiza, as was already pointed out by several authors (Gogala 1991; Barker and Tagu 2000;

Martin et al. 2001). Indeed in our experiments increased colonisation with mycorrhizal fungus was observed in ZR treated spruce seedlings. In experiments of Martin et al. (2001) treatment with zeatin changed the morphology of the hyphae of *P. tinctorius* and triggered enhanced accumulation of hyphaphorine, which plays an important role in the formation of the ectomycorrhizal root (Ditengou et al. 2000). Other studies have shown that cytokinins are able to supress host cell death in infected tissues, thereby allowing fungal development and growth within healthy tissue (Murphy et al. 1997). Furthermore it was confirmed that biotrophic and hemibiotrophic fungal pathogens are able to release the active forms of cytokinins from the pool of inactive *O*-glucosides (Cooper and Ashby 1998), thus influencing the host balance of growth regulators (Walters and McRoberts 2006). In contrast to biotrophic and hemibiotrophic fungal pathogens, no production of cytokinins or O-glucoside cleaving enzymes was observed in fungal necrotrophs (Cooper and Ashby 1998). In our experiments the colonisation levels of spruce seedlings with *H. annosum* were not affected by the applied ZR. Furthermore ZR treated *H. annosum* showed a decreased in colony growth at higher ZR concentrations, which seems to apply also to other necrotrophic fungi (Michniewicz et al. 1984).

Treatment with elicitors from both fungi increased content of soluble proteins and POD activity in the roots of spruce seedlings, whereas increased PAL activity, accompanied by accumulation of soluble phenolics and free SA, was observed only in seedlings treated with elicitors of *H. annosum*. Increased PAL and POD activity is often reported for Norway spruce treated with elicitors or after inoculation with ectomycorrhizal and pathogenic fungi (Asiegbu et al. 1994; Mensen et al. 1998; Nagy et al. 2004, Likar and Regvar, 2008) and is associated with accumulation of phenolics with function in wall strengthening (Hammerschmidt 1999). In Norway spruce seedlings the inoculation with *H. annosum* and *P. tinctorius* increased activity of guiaicol POD and ferulic acid POD and induced expression of new POD isoforms (Likar and Regvar 2008). Increased POD activity and induction of POD isoforms was observed also after treatment with the cell preparations of both fungi, suggesting increased cell-wall strengthening (Cahill and McComb 1992), and possibly formation of POD-generated fungitoxic compounds. Inhibitory effects of ZR on the POD activity observed in our present experiment, could thus severely affect the response of *P. abies* seedling to the colonisation by the tested fungi. Indeed, Beckman and Ingram (1994) showed that exogenous kinetin is able to inhibit hypersensitive response in potato inoculated with *Phytophtora infestans*, which could be due to inhibition of the apoplastic peroxidases (Bolwell et al. 2002). Cytokinins are known to regulate the expression of acid peroxidases (Limam et al. 1998), which play an important role in cell wall strengthening (Chitoor et al. 1997; Chitoor et al. 1999). Increased levels of soluble phenolics in ZR treated spruce seedlings in our experiments in combination with decreased POD activity could suggest accumulation of phenolic precursors destined for polymerisation in the cell wall, thus leading to improved conditions for fungal penetration and formation of ectomycorrhizae. Production of cytokinins in several ectomycorrhizal fungi (Kraigher et al. 1991) and improved mycorrhization of Norway spruce seedling after treatment wit ZR as observed by Gabrovšek and Gogala (1995) could support this hypothesis.

In contrast to POD activity, PAL activity and free SA accumulation, was increased only in seedling treated with *H. annosum* cell wall preparation. In addition to synthesis of precursors of several cell wall compounds, PAL can also play an important role in SA synthesis (Mauch-Mani and Slusarenko 1996) and as such in spreading the defence activation signal throughout the plant (Ryals et al. 1996). A positive correlation between SA and PAL activity, together with the accumulation of a SA precursors in the phenylpropanoid pathway was seen in *H. annosum*-inoculated spruce seedlings (Likar and Regvar 2008). As the synthesis of SA through the PAL pathway is linked to plant-cell death (Wildermuth et al. 2001), it was suggested that *H. annosum* exploits the plant-cell death for facilitation of its infection (Likar and Regvar 2008), as was observed also for *Botrytis cinerea-Arabidopsis thaliana* interactions (Govrin and Levine 2000). In our experiments, ZR treatment did not affect either PAL activity or free SA accumulation after the treatment with the elicitors and thus had no effect

on the induction of hypersensitive reaction. Based on our observations and the absence of cytokinin production in necrotrophic fungal pathogens, we assume that in contrast to biotrophic fungi, the cytokinins are not involved in the pathogenesis of the necrotrophs.

In conclusion, stimulating effects of ZR on *P. tinctorius* (growth, ergosterol concentrations) and improved colonisation of spruce seedlings, suggest that cytokinins can successfully alter the growth and colonisation success of mycorrhizal fungal symbiont. Effects of ZR on cell-wall strengthening responses after elicitation with elicitors of both fungi point to involvement of cytokinins in non-specific defence responses. In combination cytokinins could play an important role in regulation of cell wall modifications during the fungal colonisation and formation of ectomycorrhizae, by simultaneously affecting fungal growth and physiology, as well as the non-specific defence reactions of the plant host. In comparison with the ectomycorrhizal fungus *P. tinctorius*, elicitors of necrotroph *H. annosum* activated also PAL activity and the SA-dependant signal pathway, which showed no ZR-induced changes. Growth of the fungus in axenic culture and colonisation of spruce seedlings with the pathogen were not improved as in the case of the ectomycorrhizal fungus, suggesting a minor role of cytokinins in the pathogenesis of *H. annosum* on spruce.

Povzetek

Rastline s korenin sproščajo kompleksno mešanico spojin, ki tvorijo koreninski eksudat. Pole aminokislin, organskih kislin, proteinov in sladkorje, koreninski eksudat vsebuje tudi številne rastne regulatorje kot so npr. citokinini (Neumann in Römheld 2000). Citokinini igrajo pomembno vlogo pri regulaciji številnih procesov tekom rasti in razvoja rastlin (Sakakibara 2006, Kyozuka 2007), hkrati pa lahko vplivajo tudi na rast gliv (Barker in Tagu 2000, Nasim in Rehman 2006).

Iz literature je znano, da lahko citokinini vplivajo tudi na obrambne reakcije rastlin, ki se sprožijo po aplikaciji elicitorjev ali ob inokulaciji z živo glivo (Kadioglu in Durmus 1997, Limam et al. 1998, Sano et al. 1996). V pričujoči raziskavi smo, za ovrednotenje njihove vloge pri regulaciji kolonizacije kalic smreke (*Picea abies*) z mikoriznimi in patogenimi glivami, preverili vpliv zeatin ribozida (ZR): i) na rast ektomikorizne glive *Pisolithus tintorius* in patogena *Heterobasidion annosum* v aksenični kulturi, ii) na stopnjo kolonizacije kalic smreke z obema glivama in iii) na aktivacijo obrambnih reakcij smreke po tretiranju z elicitorji.

V aksenični kulturi smo pri 10^{-2} µM koncentraciji ZR opazili pospešeno rast mikorizne glive *P. tinctorius*, ki jo je spremljala povečana koncentracija ergosterola v miceliju. Nasprotno je bila rast patogene glive *H. annosum* pri najvišji koncentraciji ZR v gojišču (10 µM) zavrta. Podobno kot v aksenični kulturi je dodaten ZR pospešil kolonizacijo kalic smreke z ektomikorizno glivo, medtem ko na stopnjo kolonizacije s patogenom ni imel učinka. Tretiranje smreke z elicitorji obeh gliv je povečalo aktivnost peroksidaz (POD) v koreninah kalic, samo elicitorji patogene glive *H. annosum* pa so povečali tudi aktivnost fenilalanin amonijeve liaze (PAL) in koncentracijo topnih fenolov ter proste salicilne kisline (SA). Dodatek ZR je znižal peroksidazno aktivnost v kalicah tretiranih z elicitorji obeh gliv in povečal koncentracijo topnih fenolov.

Na podlagi naših rezultatov predvidevamo, da je ZR vpleten v regulacijo modifikacij celične stene ob glivni kolonizaciji z ektomikorizno glivo *P. tinctorius* in vzpostavitev ektomikorize, preko delovanja na glivnega partnerja in nespecifične obrambne reakcije gostitelja.

Povečana aktivnost PAL in aktivacija od SA odvisne signalne poti sta se izkazali za neobčutljive za dodatek ZR. Podobno dodan ZR ni imel pozitivnega vpliva na rast nekrotrofa *H. annosum* v aksenični kulturi ali na stopnjo kolonizacije kalic smreke, kar nakazuje, da imajo citokinini zanemarljivo vlogo pri patogenezi smreke s tem patogenom.

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Wet meadows with Purple Moor-grass (*Molinia caerulea)* in Slovenia

Mokrotni travniki z modro stožko (*Molinia caerulea*) *v Sloveniji*

Igor Zelnik

University of Ljubljana, Biotechnical Faculty, Department of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia.
*correspondence: igor.zelnik@bf.uni-lj.si

Abstract: The paper presents wet meadow vegetation with taxon *Molinia caerulea* (L.) Moench subsp. *caerulea* in Slovenia. The main objective of this study was to examine the plant species composition and plant species richness of wet meadow plant communities with the mentioned dominating or co-dominating plant taxon. Vegetation was recorded in accordance with standard Central European method. Vegetation types were classified by means of multivariate analysis. Four associations from the alliance *Molinon* Koch 1926 were identified and analysed: *Plantagini altissimae-Molinietum caeruleae* Marchiori & Sburlino 1982, *Selino-Molinietum caeruleae* Kuhn 1937, *Carici davallianae-Molinietum caeruleae* Špániková 1978 and *Junco-Molinietum caeruleae* Preising 1951 ex Klapp 1954. Ecological characteristics, plant species composition and richness of the delimited plant communities are presented, as well as their syntaxonomic position and distribution. For two of the mentioned communities relevés made in Slovenia are published here for the first time.

Keywords: *Molinia caerulea* (L.) Moench subsp. *caerulea,* plant species composition, vegetation ecology, wetlands

Izvleček: V prispevku je predstavljena vegetacija mokrotnih travnikov s taksonom *Molinia caerulea* (L.) Moench subsp. *caerulea* v Sloveniji. Glavni cilj je bil preučiti floristično sestavo in vrstno pestrost rastlinskih združb na mokrotnih travnikih z omenjenim dominantnim ali ko-dominantnim taksonom. Vegetacijo smo popisali po standardni srednjeevropski metodi. Vegetacijske tipe smo uvrstili v sintaksonomski sistem s pomočjo multivariatnih analiz. Določili in analizirali smo štiri asociacije iz zveze *Molinon*: *Plantagini altissimae-Molinietum caeruleae* Marchiori & Sburlino 1982, *Selino-Molinietum caeruleae* Kuhn 1937, *Carici davallianae-Molinietum caeruleae* Špániková 1978 in *Junco-Molinietum caeruleae* Preising 1951 ex Klapp 1954. Predstavljene so ekološke značilnosti, floristična sestava in vrstna pestrost navedenih rastlinskih združb kot tudi njihov sintaksonomski položaj in razširjenost. Za dve rastlinski združbi popise, narejene v Sloveniji, tukaj objavljamo prvič.

Ključne besede: *Molinia caerulea* (L.) Moench subsp. *caerulea,* floristična sestava, vegetacijska ekologija, mokrišča

Introduction

Wet meadows and other similar wetland-types have been the object of several studies throughout Europe in the last decade (Hájek and Hájková 2004, Havlová et al. 2004, Hölzel and Otte 2004, Hrivnák 2004, Grootjans et al. 2005, Stančić 2005, Zelnik 2005a,b, Hájková et al. 2006, Havlová 2006, Řezníčková 2007, Janišová et al. 2007, Stančić 2008, Zelnik and Čarni 2008a,b), since the threat to the biodiversity of these ecosystems is still increasing and numerous sites have been destroyed, respectively.

Meadows of the alliance *Molinion* Koch 1926 are found in nutrient-poor soils (Hölzel and Otte 2004), which may dry up during the summer (Botta-Dukát et al. 2005). They thrive on permanently to alternatively wet soils, which are acid, or alkaline (Ellmauer and Mucina 1993).

These oligotrophic ecosystems need management, which should be neither excessive nor lacking in its intensity (Ellmauer and Mucina 1993). In the last decades a decrease in biodiversity of wet meadows due to intensified agricultural use, namely the use of fertilizers has been detected (Ellmauer and Mucina 1993, Joyce 2001, McCrea et al. 2001).

Knowledge of the plant communities enables us to forecast the likely changes in floristic composition after changes of site factors (Grevilliot and Muller 2002). Nutrient availability and water regime are considered to be the most important factors determining the structure and properties of wet grassland vegetation (De Mars et al. 1996, van Duren and Pegtel 2000, Zelnik and Čarni 2008a).

Horvatić (1939) was the first researcher who systematically studied vegetation of the wet meadows in Slovenia. During the study of the vegetation of the lake Cerkniško jezero Ilijanić (1979) described new plant association *Deschampsio-Plantaginetum altissimae*, and classified it to the alliance *Molinion*. He recorded stands of this vegetation type in Planinsko polje as well. Vegetation with *Molinia caerulea* in Ljubljana moor was studied by Seliškar (1986). Vegetation of the lake Cerkniško jezero was studied also by Martinčič (1991, 2001), who described new plant associations *Schoeno nigricantis-Molinietum caeruleae* Martinčič 1991 and *Schoeno ferruginei-Molinietum caeruleae* Martinčič 2001 and classified them to the alliance *Molinion*. This type of vegetation on the Bloke plateau and in some other areas in Dinaric and Alpine regions was studied by Leskovar (1996). Kaligarič (1997) documented the thriving of the association *Selino-Molinietum caeruleae* Kuhn 1937 near Slovenj Gradec. Results of the studies of the wet meadows in SE Slovenia (Krško basin, Bela Krajina) are published in Zelnik (2005a), Zelnik and Čarni (2008b). Vegetation ecology of some of the communities from the alliance *Molinion* in Slovenia is discussed in Zelnik and Čarni (2008a).

Beside the mentioned publications that cover only some of the areas in Slovenia, wet meadow vegetation in major part of the country remained almost unknown to the scientific public, especially the presence, structure and distribution of several plant communities. One of the aims of this paper is to fill this gap and to present the characteristics and distribution of wet meadow plant communities with *Molinia caerulea* (Purple Moor-grass) in Slovenia. The aims of this study were also to examine:

– The structure and diversity of wet meadow plant communities with dominant taxon *Molinia caerulea* ssp. *caerulea*.
– Relationships between wet meadow communities of *Molinon* alliance in Slovenia and their distribution.

Material and methods

Study area

The meadows were investigated across the majority of the state, in the continental part of Slovenia from the western border of Dinaric region to the eastern border of the state, which is in the Pannonian region (from 14˚10’ to 16˚20’E, from 45˚40’ to 46˚50’N). There is a strong gradient in annual precipitation from the SW to NE part of the studied area. In the SW part the climate is more humid with an annual precipitation of 1500 mm, but in the NE part, which is the driest part of Slovenia the annual precipitation is 800 mm (Zupančič 1995). For this study vegetation plots from NE, E and SE parts of the state were excluded from analysis due to absence of the taxon *Molinia*

caerulea ssp. *caerulea*. Moreover, only stands with dominant or co-dominant mentioned taxon were considered for this study. There is also an altitudinal gradient which reflects in mean annual temperatures, as some plots can be found at about 265 m and others 680 m a.s.l., but the majority is found between 300 and 500 m a.s.l.

Vegetation analysis

Vegetation was investigated according to the standard Central European method (Braun-Blanquet 1964). The cover-abundance values were transformed according to van der Maarel (1979). Vegetation relevés (59) were made in the years 2003 and 2004. The size of plots varies from 15 to 25 m^2 due to micro-topography. Nomenclature of plant taxa follows Ehrendorfer et al. (1973) with exception of taxa *Centaurea macroptilon* Borb., *Centaurea carniolica* Host.

Similarity analyses of the relevés were carried out using the computer program SYN-TAX (Podani 2001); an ordination method (PCoA) was performed. Dissimilarity of relevés was measured with Similarity ratio complement. Rare species were not excluded from the analysis. Clusters of relevés were classified into syntaxa according to Ellmauer and Mucina (1993) as well as local studies (Zelnik 2005a,b).

Results and discussion

Floristically defined wet meadow communities

Since the objects of our study were stands with abundant taxon *Molinia caerulea* ssp. *caerulea*, the classification to the alliance *Molinion* could be done without any doubt. Further classification of the relevés was done on the base of the ordination diagram (Fig. 1), which shows the grouping of the relevés according to the similarity of their floristic composition. The four groups corresponded to four wet meadow plant associations belonging to *Molinion* alliance and were classified according to Ellmauer and Mucina (1993), Sburlino et al. (1995), Zelnik (2005a,b), Zelnik and Čarni (2008a): (1) *Plantagini altissimae-Molinietum caeruleae* Marchiori & Sburlino 1982, (2) *Selino-Molinietum caeruleae* Kuhn 1937, (3) *Carici davallianae-Molinietum caeruleae* Špániková 1978 and (4) *Junco-Molinietum caeruleae* Preising 1951 ex Klapp 1954.

Despite the results of formalized classification of the stands with *Molinia caerulea*, that were obtained in several countries and are able to define only two different associations, we disagree with such simplification of this diverse vegetation in floristic and ecological sense that reflects in high species diversity and diversity of communities/ecosystems. Statistically significant differences in many measured and/or calculated ecological parameters between these associations were calculated and published (Zelnik and Čarni 2008a), so these clearly defined associations from the alliance *Molinion* obviously exist.

Syntaxonomical scheme of the studied vegetation:

Molinio-Arrhenatheretea R.Tx. 1937 em. R. Tx. 1970
Molinietalia Koch 1926
Molinion Koch 1926
Plantagini altissimae-Molinietum caeruleae Marchiori & Sburlino 1982
Selino-Molinietum caeruleae Kuhn 1937
Carici davallianae-Molinietum caeruleae Špániková 1978
Junco-Molinietum caeruleae Preising 1951 ex Klapp 1954

The sampling plots in the ordination diagram were segregated according to communities (Fig. 1) and also correspond to the traditional method:

- *Plantagini-Molinietum* with the highest scores along the first axis (the most humid climate) and intermediate along the second axis;
- *Selino-Molinietum* with intermediate position that is in accordance with its central position within the alliance *Molinion*;
- *Carici-Molinietum* with the highest scores along the first axis (the highest pH) and moderate humidity;
- *Junco-Molinietum* with high scores along first axis (humid sites – depressions);

Their characteristic taxa are presented in Table 1, while their plant species composition is presented in Tables 2 and 3. In total 232 plant taxa were found in 59 sampled plots, ranging from 14 to 69 plant taxa per plot.

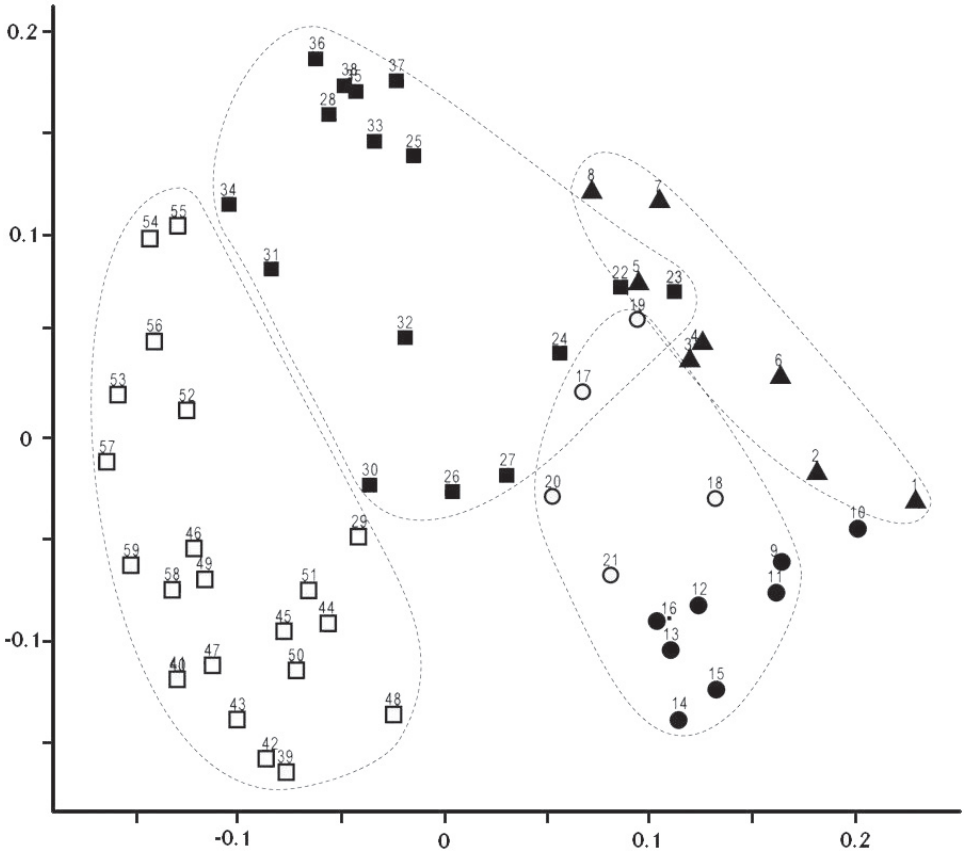

Figure 1: Ordination diagram of sampling plots based on analysis PCoA. Numbers correspond to the numbers of relevés in Tables 2-3 (complement of similarity ratio).

Slika 1: Ordinacijski diagram popisov na osnovi metode PCoA. Številke so v skladu s številkami popisov v Tabelah 2-3 (komplement koeficienta podobnosti).

▲ 1–8: *Plantagini altissimae-Molinietum caeruleae*,
● 9–16: *Junco-Molinietum caeruleae succiselletosum inflexae*,
○ 17–21: *Junco-Molinietum caeruleae typicum,*
■ 22–38: *Selino-Molinietum caeruleae,*
□ 39–59: *Carici davallianae-Molinietum caeruleae.*

***Plantagini altissimae-Molinietum caeruleae* Marchiori & Sburlino 1982**

This association was described in eastern parts of Po plain (Veneto, Friuli), where it thrives in helocrenic spring sites and in higher parts of the lowland (Sburlino et al. 1995). Stands often occur in depressions within intensively cultivated areas, where the mineralization of soil organic matter is hindered due to high water content (Sburlino et al. 1995).

The association *Plantagini altissimae-Molinietum caeruleae* Marchiori & Sburlino 1982 is found in the western part of studied area, where the climate is most humid and this is the main ecological difference with communities of the alliance *Deschampsion* Horvatić 1930, which thrive in the areas with more arid climate.

Stands classified to this association were recorded in the valley of river Nanoščica. They thrive in bigger depressions, which do not lie

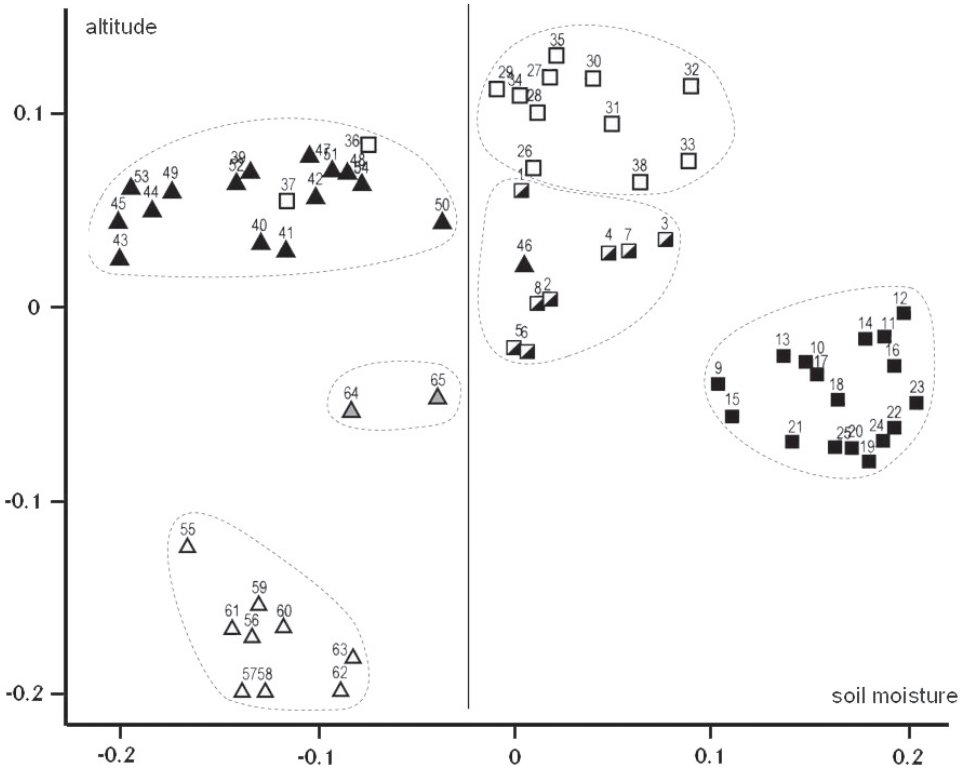

Figure 2: Ordination of the relevés of the associations *Plantagini altissimae-Molinietum caeruleae* and *Deschampsio-Plantaginetum altissimae* (PCoA, complement of similarity ratio):

Slika 2: Ordinacija popisov asociacij *Plantagini altissimae-Molinietum caeruleae* in *Deschampsio-Plantaginetum altissimae* (PCoA, komplement koeficienta podobnosti):

- ◪ 1–8:Zelnik, hoc loco, tab. 2 / 1–8 *Plantagini altissimae-Molinietum caeruleae*;
- ■ 9–25: Sburlino et al. (1995), tab. 1 / 1–17 *Plantagini altissimae-Molinietum caeruleae*;
- □ 26–37: Ilijanić (1979), tab. 11 / 1–12 *Deschampsio-Plantaginetum altissimae cirsietosum pannonici;*
- ▲ 38–54: Ilijanić (1979), tab. 11 / 13–29 *Deschampsio-Plantaginetum altissimae typicum & filipenduletosum vulgaris;*
- △ 55–63: Zelnik, 2005b, tab. 6 / 1–9 *Deschampsio-Plantaginetum altissimae caricetosum tomentosae;*
- ◬ 64–65: Zelnik, 2005b, tab. 6 / 10–11 *Deschampsio-Plantaginetum altissimae molinietosum arundinaceae;*

along the mentioned river on its floodplain, but along its tributaries that drain the areas on non-calcareous bedrock.

Characteristic species and plant species composition: Sburlino et al. (1995) declared the species *Plantago altissima* as the only characteristic species with sufficient constancy. Additionaly, species *Centaurea carniolica* is differential from other central European communities with *Molinia*.

Beside the mentioned species we name the following species as differential from other stands with *Molinia* (Tab. 2, relevés 1–8): *Sanguisorba officinalis, Gratiola officinalis, Cirsium rivulare.*

From similar association *Deschampsio-Plantaginetum altissimae* this association could

be told apart on the base of the *Molinia caerulea*, which is often dominant species here, but missing in the mentioned. Besides, this association could be delimited from the mentioned due to presence of the following species: *Serratula tinctoria, Carex nigra, Ranunculus flammula, Potentilla erecta*.

Syntaxonomic position and distribution in other countries: On the base of the comparison of the relevés we discovered the similarity of this association with the syntaxon *Deschampsio-Plantaginetum altissimae cirsietosum pannonici* Ilijanić 1979, which thrives on the Cerkniško jezero. Ilijanić (1979) had already pointed out the possibility of delineation of this subassociation and definition as specific association. Multivariate analyses revealed relatively higher similarity of the mentioned subassociation with *Plantagini altissimae-Molinietum caeruleae* than other units of the *Deschampsio-Plantaginetum altissimae* (Fig. 2). According to these findings we classified mentioned subassociation and our stands into association *Plantagini altissimae-Molinietum caeruleae*. However, stands from northern Italy are richer in fen species, so on the base of our data and additional research there is a possibility to describe a distinct subassociation in the future.

The only report about the thriving of this association outside Po plain is given by Zelnik and Čarni (2008a).

Selino-Molinietum caeruleae Kuhn 1937

For this central association of the alliance *Molinion* many authors use the name *Molinietum caeruleae*, or *Molinietum medioeuropaeum* Oberdorfer 1957, which are not clearly defined. For this reason Ellmauer and Mucina (1993) suggest the name *Selino-Molinietum* Kuhn 1937 for such stands.

This is basophilic community that thrives in lowland and montane belt, mostly in less wet fen soils. Soils are almost equally wet throughout the whole year. Stands are mown in late summer or early autumn once a year or biennially. This community is a stage in succession of aquatic ecosystems to terrestrial leading from tall-sedge communities of the alliance *Magnocaricion elatae* Koch 1926, low-sedges from the alliance *Caricion davallianae* Klika 1934 to the community *Selino-Molinietum* (Ellmauer and Mucina 1993). This association can also develop on the edge of bog or with degradation of fens.

First relevés from Slovenia that were classified into this association are from the vicinity of Slovenj Gradec and were published by Kaligarič (1997). Our relevés from various sites were classified into this association, which is in accordance with its central position and wide distribution (Tab. 3, relevés 22–37).

Characteristic species and plant species composition: This is a central community of the alliance *Molinion* that reflects in high constancy of characteristic species of this alliance.

In accordance with other authors (Ellmauer and Mucina 1993, Kaligarič 1997), we pointed out the following species as characteristic: *Selinum carvifolia, Laserpitium prutenicum.*

Syntaxonomic position and distribution in other countries: Since this is a central association of the alliance it similarity to the other associations is relatively high. Community was described in southern Germany by Kuhn (1937), but many authors classified more or less similar stands to macro-association *Molinietum caeruleae*, or *Molinietum medioeuropaeum*, that prevented a review. There is confusion in literature, since some authors classify different associations or their parts into *Selino-Molinietum* (Pott 1995). Even in the latest publications (Burkart et al. 2004, Havlová 2006, Janišová et al. 2007) this vegetation is classified as *Molinietum* Koch 1926.

The reasons for this is the difficulty of classification of the stands with *Molinia caerulea*, the lack of knowledge of the ecological conditions of these sites and the absence of multivariate analyses that would enable comparisons of plant species composition with relevés of other associations.

Another reason is the abandonment of mowing of the meadows, where stands with *Molinia caerulea* thrive that leads to the total dominance of this species, while others e.g. characteristic species of many associations disappear from the stands and make the suitable classification impossible. The consequence is the classification of such stands to the most intermediate association *Selino-Molinietum*.

Distribution of this association is documented in Germany (Kuhn 1937, Pott 1995), Austria (Balátová-Tuláčková and Hübl 1985a,

Ellmauer and Mucina 1993) and Italy (Sburlino et al. 1995).

Carici davallianae-Molinietum caeruleae Špániková 1978

This community that represents a transition between wet meadows and fens is dominated by taxon *Molinia caerulea ssp. caerulea* and is often adjacent to the stands of *Caricetum davallianae* Dutoit 1924 (Špániková 1978), but its floristic composition is still richer in meadow species, which also dominate the stands.

These stands are often found in helocrenic springs that are surrounded by mesic communities. Soils are loamy and often with high share of organic matter. Soils are slightly acid to neutral, however, pH values and humidity are higher than in *Selino-Molinietum*. This association thrives in permanently wet sites rich in Ca^{2+}. These conditions reflect in higher share of the fen species. Since the soils are rarely dry enough, mowing with tractors is barely possible in these meadows that was evident in the field. With the lowering of water level the vegetation turns to the association *Selino-Molinietum*.

In all studied sites the influence of base-rich groundwater was obvious. Moreover, this water drains the limestone and/or dolomite and supplies these sites with high amounts of Ca^{2+} and Mg^{2+}. These sites are never on plane, but on slight slopes (3–10°). Stands of this association were recorded in Alpine, Pre-Alpine, Dinaric and Pre-Dinaric phytogeographic regions of Slovenia, on the following locations: Dolič, Sopota, Tatinec, Nemška vas.

Characteristic species and plant species composition: On the base of the comparison of our stands (Tab. 3, relevés 38–59) with stands of other associations, we defined (in accordance with Dierschke 1994) the following species as differential: *Carex davalliana, C. hostiana, Epipactis palustris, Koeleria pyramidata, Eriophorum latifolium*. Mentioned species are basophilic and except *Koeleria pyramidata* are all fen species. They mostly match with the differential species of the subassociation *Selino-Molinietum caricetosum davallianae* Balátová-Tuláčková & Hübl 1985 (*Carex davalliana, C. flava, Eriophorum latifolium, Parnassia palustris*), that was described in eastern limestone Alps by Balátová-Tuláčková and Hübl (1985a). In the same publication these authors defined the same combination of the species (*Carex davalliana, Eriophorum latifolium, Parnassia palustris)* as differential for the subassociation *Molinietum caeruleae caricetosum davallianae* Görs 1951.

Syntaxonomic position and distribution in other countries: Unclear classification of this basophilic vegetation in Slovenia was firstly pointed out by Kaligarič (1997). Authors (e.g. Seliškar 1986, Leskovar 1996) have classified such stands with stands of different other associations (e.g. *Selino-Molinietum, Plantagini altissimae-Molinietum caeruleae)* into same syntaxon *Molinietum caeruleae* s.l. Figure 1 presents clear delimitation of these stands from relevés of other associations.

Studied association is similar to *Succiso-Molinietum caeruleae* (Kovacs 1962) Soó 1969 that thrives in Pannonian region, namely in Austria (Ellmauer and Mucina 1993) and Hungary (Borhidi 2003). In case of increasing moisture of those sites Wagner (1950) defined its transition to community *Schoenetum nigricantis* Koch 1926. In our case increasing moisture of the sites led to the fen community *Caricetum davallianae* Dutoit 1924.

Our relevés are very similar to the relevés published by Balátová-Tuláčková and Hübl (1985a) that are recorded in the Lower-Austrian limestone Alps, where the bedrock is actually the same as in our cases. Mentioned authors classified those stands into *Selino-Molinietum caricetosum davallianae* Balátová-Tuláčková & Hübl 1985 and *Molinietum caeruleae caricetosum davallianae* Görs 1951. Our relevés are also similar to the relevés from northern Italy (Lombardy) published by Sburlino et al. (1995: Tab. 2 / 1–9).

According to the mentioned facts and similarities we can conclude that this basophilic community is distributed over Slovenia and in other countries that reach into the southern and/or northern belt of calcareous Alps, Carpathians and NW Dinaric mountains.

Junco-Molinietum caeruleae Preising 1951 ex Klapp 1954

Soils in this community are of loamy texture and could be gleysols (Balátová-Tuláčková and

Hübl, 1985b) and/or peat-soils. These sites are rarely fertilized and mown only once a year (Ellmauer and Mucina 1993). Sites are mostly on intermittently wet riverine soils or slopes with helocrenic springs (hanging mires). This association is common in areas with non-carboniferous bedrock and/or in wet places with thick layer of accumulated organic matter (peat).

In Slovenia this association is most common in Ljubljana moor, where it was documented by Seliškar (1986). Stands of this association were recorded on the following localities (Tab. 2, relevés 9–21): Nemška vas, Log pri Mokronogu, Selo pri Bledu. Soils under the stands on the mentioned localities are influenced by the base-poor water that drains non-calcareous sediments and/or are in depressions where rainwater is stagnating for weeks.

Characteristic species and plant species composition: Association is poorly floristically defined. Characteristic species of the order *Molinietalia Koch 1926* as well as of the alliance *Molinion* are relatively rare. Beside the mentioned characters, numerous species characteristic for the classes *Scheuchzerio-Caricetea fuscae* R.Tx. 1937 and *Calluno-Ulicetea* that indicate nutrient-poor acidic soils, were found in these stands.

Preising (1951), Ellmauer and Mucina (1993) defined species *Juncus conglomeratus* and *J. effusus* as differential from other associations.

Syntaxonomic position and distribution in other countries: After Ellmauer and Mucina (1993) this community is transitional towards following syntaxa: *Calthion*, *Caricion fuscae* (*Scheuchzerio-Caricetea fuscae*) and *Violion caninae* (*Calluno-Ulicetea*).

Such stands are found in Austria on silicate bedrock on the altitudes between 525 and 660 m a. s. l. (Balátová-Tuláčková and Hübl 1985b). Association thrives also in Hungary (Borhidi 1996), what is no longer evident in later publications (Borhidi 2003), since the name *Nardo-Molinietum hungaricae* is used for this type of vegetation. Association is also found in Czech Republic (Blažková 1973, Havlová 2006) and in Germany (Pott 1995), although in later publications (Burkart et al. 2004) these stands are classified to community *Juncus conglomeratus-Succisa pratensis*.

Division to lower syntaxa: Balátová-Tuláčková and Hübl (1985b) classified relevés from Austria into subassociations *scirpetosum sylvaticae* Balátová-Tuláčková & Hübl 1985 and *typicum* Preising 1951.

Junco-Molinietum caeruleae typicum Preising 1951: Five our relevés, without differential species, were classified into typical subassociation (Tab. 2, relevés 17–21). Prevailing species in these stands are species of the order *Molinietalia* and class *Molinio-Arrhenatheretea*.

We defined new subassociation *succiselletosum inflexae* due to dominance of the species *Succisella inflexa* (Tab. 2, relevés 9–16; *holotipus*: Tab. 2 / relevé 13).

Junco-Molinietum caeruleae succiselletosum inflexae subass. nova hoc loco: On the bottoms of more distinctive depressions (> 0,2 m), beside the species *Molinia caerulea, Succisella inflexa,* that is characteristic species of the alliance *Deschampsion* Horvatić 1930, is very abundant. In such depressions soils are under influence of stagnating base-poor rainwater that makes the ecological conditions similar to the ones where communities of the alliance *Deschampsion* are found. Main difference in floristic composition is in presence and even dominance of the species *Molinia caerulea*.

Because of the mentioned conditions lower herb layer is dominated by species *Succisella inflexa* and *Carex panicea*, while upper is dominated by *Molinia caerulea*. Other species only exceptionally occur with higher cover values, their number is much lower than in other communities with *Molinia caerulea*. From other classes marsh species of the class *Phragmito-Magnocaricetea Klika 1941* can be found in these stands.

Such stands were recorded in the area of Alpine-Dinaric mountain barrier where the precipitation amount is the highest in Slovenia. These localities were: Nemška vas near Ribnica, Selo pri Bledu.

These stands thrive in sites that are wetter than sites of other communities like *Junco-Molinietum caeruleae typicum, Plantagini altissimae-Molinietum*, what reflects in plant species composition (Fig. 1). Further increasing of soil moisture on lower sites facilitates species of the classes *Phragmito-Magnocaricetea* and *Scheuchzerio-Caricetea fuscae* that become dominant.

Conclusions

Vegetation of wet meadows in the research area is very diverse. Our relevés with taxon *Molinia caerulea* subsp. *caerulea* were classified into alliance *Molinion* and further into four plant associations: *Plantagini altissimae-Molinietum caeruleae* Marchiori & Sburlino 1982, *Carici davallianae-Molinietum caeruleae* Špániková 1978, *Selino-Molinietum caeruleae* Kuhn 1937, *Junco-Molinietum caeruleae* Preising 1951. For the first two associations the relevés from Slovenia are published for the first time in analytical table in this paper. Beside the mentioned four associations another two vegetation types with dominant or co-dominant Molinia caeruleae that are transitional to fens are found in Slovenia, namely *Schoeno nigricantis-Molinietum caeruleae* Martinčič 1991 and *Schoeno ferruginei-Molinietum caeruleae* Martinčič 2001. Since they occur on the margins of the fens which distribution is very limited (*Schoenetum nigricantis* Koch 1926, *Schoenetum ferruginei Du Rietz 1925*) only, they are not widely distributed and are not studied in this paper.

The association *Plantagini altissimae-Molinietum caeruleae* Marchiori & Sburlino 1982 is found in the western part of studied area, where the climate is most humid and this is the main ecological difference with communities of the alliance *Deschampsion*, which thrive in the areas with more arid climate. This association is found also in northern Italy (Sburlino et al. 1995).

The association *Selino-Molinietum caeruleae* Kuhn 1937 is the central association of the alliance *Molinion* and corresponds to the community *Molinietum caeruleae* Koch 1926 (Ellmauer and Mucina 1993, Zelnik 2005b), which is not efficiently defined. This community is found all over central Europe.

The association *Carici davallianae-Molinietum caeruleae* Špániková 1978 is transitional to fen vegetation, but its floristic composition is still much richer in meadow species, which also dominate the stands. This association is found in central and north Slovenia (Zelnik 2005b).

Apart from the other three associations which are more or less basophilic, the association *Junco-Molinietum caeruleae* is acidophilic and is found in depressions with stagnating rainwater or on peat-soils. This association is found in central, southeast and east Slovenia (Zelnik 2005a,b).

Most species-rich plant communities thrive in sites without any outstanding parameter that would have a dominating influence on the conditions. It is crucial that soils are nutrient-poor and flooded/waterlogged for short periods only.

Slovenia is in conjunction of Alpine, Mediterranean, Dinaric and Pannonian regions that reflects in diversity of wet meadow plant communities. We found plant communities that were described in Germany, Italy and Slovakia, and are distributed also in Austria, Hungary, Croatia, Czech Republic and Poland.

Povzetek

Mokrotni travniki so bili v zadnjem desetletju v Evropi pogosto predmet preučevanj, predvsem zaradi vedno večje ogroženosti njihove biodiverzitete, marsikje pa so ti ekosistemi že uničeni. Mokrotne travnike iz zveze *Molinion* Koch 1926 najdemo na tleh, ki so revna s hranili in so stalno ali občasno mokra. Za ohranjanje teh oligotrofnih ekosistemov je potrebno gospodarjenje, ki ne sme biti preveč ali premalo intenzivno.

Vegetacijo mokrotnih travnikov so v Sloveniji preučevali: Horvatić (1939), Ilijanić (1979), Seliškar (1986), Martinčič (1991, 2001), Leskovar (1996), Kaligarič (1997). Rezultati teh raziskav v zadnjem desetletju pa so objavljeni v: Zelnik (2005a), Zelnik in Čarni (2008a,b).

V Sloveniji je struktura rastlinskih združb mokrotnih travnikov podrobneje raziskana in objavljena predvsem na območjih kot je Cerkniško jezero, Ljubljansko barje, Bloke in Krška kotlina, medtem ko je v ostalih območjih strokovni javnosti nepoznana. Cilj prispevka je predstavitev značilnosti, floristične sestave, vrstne pestrosti in razširjenosti rastlinskih združb mokrotnih travnikov z *Molinia caerulea* ssp. *caerulea* v Sloveniji. Vegetacijo smo popisali po standardni srednjeevropski metodi. Vegetacijske tipe smo uvrstili v sintaksonomski sistem s pomočjo multivariatnih analiz. Določili in analizirali smo štiri asociacije iz zveze *Molinon*: *Plantagini altissimae-Molinietum caeruleae* Marchiori & Sburlino 1982, *Selino-Molinietum caeruleae* Kuhn 1937, *Carici davallianae-Molinietum caeruleae*

Špániková 1978 in *Junco-Molinietum caeruleae* Preising 1951 ex Klapp 1954.

Plantagini altissimae-Molinietum caeruleae Marchiori & Sburlino 1982: Ta asociacija je bila opisana v vzhodnih predelih Padske nižine, kjer uspeva na povirnih mestih. Sestoje, ki smo jih uvrstili v to asociacijo smo popisali v dolini reke Nanoščice. Uspevajo v večjih ulekninah, ki pa ne ležijo neposredno ob Nanoščici temveč ob njenih pritokih, ki imajo prispevna območja na pretežno nekarbonatnih kamninah.

V naših popisih (Tab. 2, popisi 1–8), se poleg značilnice *Plantago altissima* kot razlikovalnice od ostalih sestojev s stožko pojavljajo naslednje vrste: *Sanguisorba officinalis, Gratiola officinalis, Centaurea carniolica, Cirsium rivulare.* Od podobne združbe *Deschampsio-Plantaginetum altissimae* se ta asociacija bistveno loči po vrsti *Molinia caerulea*, ki je tukaj pogosto dominantna vrsta in po naslednjih vrstah: *Serratula tinctoria, Carex nigra, Ranunculus flammula, Potentilla erecta*.

Selino-Molinietum caeruleae Kuhn 1937: Za to osrednjo združbo zveze *Molinion* mnogi avtorji uporabljajo ime *Molinietum caeruleae*, oziroma *Molinietum medioeuropaeum* Oberdorfer 1957, ki pa sta premalo natančno definirani. Zato Ellmauer & Mucina (1993) za tovrstne sestoje predlagata uporabo imena *Selino-Molinietum* Kuhn 1937. To je bazifilna združba, ki uspeva v nižinskem in montanskem pasu, večinoma na manj vlažnih tleh. Tla so preko celega leta enakomerno vlažna. Prve popise iz Slovenije, ki so klasificirani v to asociacijo, je objavil Kaligarič (1997).

Kot značilnice smo izpostavili naslednji vrsti: *Selinum carvifolia, Laserpitium prutenicum.*

Ker je to osrednja asociacija zveze *Molinion,* je zaradi svoje relativne zmernosti tudi najbolj podobna vsem ostalim združbam iz te zveze. Klasifikacija teh sestojev je pogosto težavna zaradi nepoznavanja rastiščnih razmer in opuščanja košnje teh površin, zaradi česar modra stožka popolnoma prevlada, ostale vrste med drugim tudi značilnice, pa izginjajo iz sestojev.

Carici davallianae-Molinietum caeruleae Špániková 1978: V tej združbi, ki predstavlja prehod med mokrotnimi travniki in nizkimi barji in je pogosto v stiku z asociacijo *Caricetum davallianae*, prevladuje vrsta *Molinia caerulea*. Sestoje te združbe vedno najdemo na povirnih mestih. Tla so ilovnata in pogosto z visokim deležem organske snovi (šotnata), reakcija tal je rahlo kisla do nevtralna, višja kot v asociaciji *Selino-Molinietum*. Na vseh rastiščih je bilo očitno, da gre za vpliv z bazami bogate talne vode, oziroma mezeče povirne vode. Ta rastišča so vedno na blagih pobočjih (3–10°).

Na osnovi naših popisov smo kot dobre razlikovalne vrste, oziroma značilnice definirali naslednje bazifilne vrste: *Carex davalliana, C. hostiana, Epipactis palustris, Koeleria pyramidata, Eriophorum latifolium*.

V preteklosti so tovrstne popise različno klasificirali in jih združevali v sintakson *Molinietum caeruleae* s.l., s popisi drugih sintaksonov z modro stožko. Na sliki 1 vidimo, da se popisi obravnavane asociacije jasno razlikujejo od ostalih. Zelo podobni našim popisom, so sestoji, ki jih navajata Balátová-Tuláčková & Hübl (1985a) z avstrijskih apneniških Alp in sestoji iz severne Italije (Sburlino in sod. 1995).

Junco-Molinietum caeruleae Preising 1951 ex Klapp 1954: Tla v tej združbi so pogosto oglejena, oziroma so šotnata. Ta asociacija je pogosta v območjih, kjer prevladuje nekarbonatna podlaga, oziroma na rastiščih, kjer je zaradi upočasnjene razgradnje nastala debela plast organskih snovi. V Sloveniji je ta asociacija pogosta predvsem na Ljubljanskem barju. Sestoje iz te asociacije smo popisali na lokacijah Nemška vas, Log pri Mokronogu in Selo pri Bledu, kjer so tla pod vplivom z bazami revne talne vode, oziroma zastajajoče deževnice.

Združba je floristično slabo definirana. Kot razlikovalnice od ostalih stožkovij navajamo vrsti *Juncus conglomeratus* in *J. effusus*.

V tipično subasociacijo smo uvrstili pet popisov (Tabela 2, popisi 17–21), v katerih nismo našli diferencialnih vrst. Zaradi dominance vrste *Succisella inflexa* v kotanjah, smo definirali novo subasociacijo *succiselletosum inflexae*.

Junco-Molinietum caeruleae succiselletosum inflexae subass. nova hoc loco: Tabela 2, popisi 9–16; *holotip*: tabela 2 / popis 13. V izrazitejših ulekninah dlje časa zastaja deževnica, zato v teh sestojih z visoko pokrovnostjo uspeva tudi vrsta *Succisella inflexa*, ki je značilnica zveze *Deschampsion*. Bistvena razlika v floristični sestavi je v uspevanju in dominanci vrste *Molinia caerulea*. Zaradi omenjenih ekstremnih razmer v spodnjem

zeliščnem sloju prevladujeta vrsti *Succisella inflexa* in *Carex panicea*, v zgornjem pa dominira vrsta *Molinia caerulea*. Poleg teh, se ostale vrste le izjemoma pojavljajo z višjimi pokrovnostmi, število vrst pa je na splošno mnogo manjše kot v ostalih stožkovjih.

Tovrstne sestoje smo našli na območju alpsko-dinarske pregrade, kjer je količina padavin največja v Sloveniji, in sicer na lokalitetah Nemška vas v Ribniški dolini in Selo pri Bledu.

Slovenija je na stičišču panonske, dinarske, alpske in sredozemske regije, kar se odraža tudi v pestrosti travniške vegetacije. V Sloveniji smo tako našli asociacije, ki so bile opisane v Nemčiji, v Italiji in na Slovaškem, uspevajo pa tudi na Hrvaškem, na Madžarskem, v Avstriji, na Češkem, na Poljskem.

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Appendix:

List of the relevé localities (from Tables 2 and 3):

Plantagini altissimae-Molinietum:

1: 0251/1 Mrzlek; **2**: 0251/1 Mrzlek; **3**: 0251/1 Mrzlek; **4**: 0251/1 Zagon; **5**: 0251/1 Mrzlek; **6**: 0251/1 Mrzlek; **7**: 0251/1 Mrzlek; **8**: 0251/1 Zagon.

Junco-Molinietum caeruleae:

9: 0254/3 Nemška vas; **10**: 0254/3 Nemška vas; **11**: 0254/3 Nemška vas; **12**: 0254/3 Nemška vas; **13**: 9650/4 Selo pri Bledu; **14**: 9650/4 Selo pri Bledu; **15**: 9650/4 Selo pri Bledu; **16**: 0254/3 Nemška vas;

17: 0254/3 Nemška vas; **18**: 0254/3 Nemška vas; **19**: 0254/3 Nemška vas; **20**: 0056/4 Log pri Mokronogu; **21**: 0056/4 Log pri Mokronogu.

Selino-Molinietum caeruleae:

22: 0054/3 Radensko polje; **23**: 0054/3 Radensko polje; **24**: 0254/3 Nemška vas; **25**: 0054/3 Radensko polje; **26**: 9650/2 Podhom; **27**: 9955/2 Sopota; **28**: 0054/3 Radensko polje; **29**: Dobje; **30**: 9556/1 Radoše; **31**: 9650/2 Podhom; **32**: 0054/3 Radensko polje; **33**: 9752/1 Tatinec; **34**: 0054/3 Radensko polje; **35**: 0054/3 Radensko polje; **36**: 0054/3 Radensko polje; **37**: 0054/3 Radensko polje.

Carici davallianae-Molinietum caeruleae:

38: 0054/3 Radensko polje; **39**: 9557/3 Dolič; **40**: 9955/2 Sopota; **41**: 9557/3 Dolič; **42**: 9557/3 Dolič; **43**: 9557/3 Dolič; **44**: 9955/2 Sopota; **45**: 9955/2 Sopota; **46**: 9955/2 Sopota; **47**: 9955/2 Sopota; **48**: 9955/2 Sopota; **49**: 9955/2 Sopota; **50**: 9955/2 Sopota; **51**: 9557/3 Dolič; **52**: 0056/3 Dol pri Trebnjem; **53**: 9752/1 Tatinec; **54**: 9752/1 Tatinec; **55**: 9752/1 Tatinec; **56**: 9752/1 Tatinec; **57**: 9752/1 Tatinec; **58**: 9752/1 Tatinec; **59**: 9752/1 Tatinec.

Community number	1	2	3	4	5
Number of relevés	8	8	5	17	21
Number of plant taxa	**47**	**23**	**37**	**39**	**33**
Plantagini altissimae-Molinietum caeruleae					
Sanguisorba officinalis	**100**	.	.	.	.
Plantago altissima	**100**	38	.	6	.
Gratiola officinalis	**100**	62	20	29	.
Centaurea carniolica	**100**	.	.	18	19
Cirsium rivulare	**75**	12	40	6	5
Serratula tinctoria	**75**	12	.	12	5
Ranunculus flammula	**62**	25	20	6	.
Carex nigra	**50**	.	20	.	.
Junco-Molinietum caeruleae succiselletosum inflexae subass. nova					
Succisella inflexa	25	**100**	.	29	.
Junco-Molinietum caeruleae typicum					
Juncus conglomeratus	100	50	**60**	35	5
Juncus effusus	25	.	**60**	6	.
Selino-Molinietum caeruleae					
Selinum carvifolia	75	.	20	**76**	.
Laserpitium prutenicum	.	.	.	**71**	.
Carici davallianae-Molinietum caeruleae					
Carex davalliana	.	.	.	12	**95**
Carex hostiana	12	50	20	71	**90**
Epipactis palustris	.	12	.	6	**90**
Koeleria pyramidata	.	.	.	24	**76**
Eriophorum latifolium	.	.	.	6	**52**
Molinion					
Molinia caerulea subsp. *caerulea*	**100**	**100**	**100**	**100**	**100**
Succisa pratensis	75	12	60	94	95
Gentiana pneumonanthe	38	62	60	65	.
Galium boreale	50	.	20	59	24
Carex distans	12	25	.	12	24
Molinia arundinacea	.	.	40	12	10
Inula salicina	.	38	.	.	5
Iris sibirica	12	.	.	18	.
Gladiolus palustris	.	.	.	6	10
Carex tomentosa	.	.	.	18	.
Gentiana asclepiadea	.	.	.	6	5
Polygala amarella	.	.	.	.	10
Ophioglossum vulgatum	12	.	.	.	.
Vicia tetrasperma	.	.	20	.	.
Molinietalia					
Filipendula ulmaria	88	88	100	53	19
Lythrum salicaria	62	100	100	29	33
Valeriana dioica	75	38	60	41	67
Lysimachia vulgaris	38	100	80	35	24
Equisetum palustre	38	88	40	18	62
Juncus acutiflorus	88	50	60	18	.
Betonica officinalis	88	.	40	71	48
Angelica sylvestris	38	.	20	35	57
Deschampsia cespitosa	38	.	40	18	5
Genista tinctoria	12	.	20	53	29
Lychnis flos-cuculi	88	.	60	6	.
Linum catharticum	25	.	.	29	33
Thalictrum lucidum	.	88	20	6	.
Myosotis scorpioides	88	12	.	12	.
Gymnadenia conopsea	.	.	.	6	52
Lotus pedunculatus	.	12	.	12	24
Senecio aquaticus agg.	50	38	.	.	.
Cirsium palustre	.	.	.	.	29
Cirsium oleraceum	.	.	.	29	33
Geum rivale	.	.	.	6	24
Cardamine pratensis agg.	12	.	20	12	.
Galium uliginosum	.	.	.	6	14
Peucedanum coriaceum	38	.	.	.	.
Crepis paludosa	25	.	.	6	.
Dactylorhiza maculata	25	.	.	6	.
Colchicum autumnale	12	.	.	12	.
Veratrum album	.	.	.	12	5
Hypericum tetrapterum	.	.	20	.	5
Scirpus sylvaticus	.	.	.	6	5
Allium angulosum	.	.	.	6	.
Caltha palustris	.	.	.	6	.

Table 1: Shortened percentage synoptic table of the studied wet meadows plant communities with *Molinia caerulea*: 1 – *Plantagini altissimae-Molinietum caeruleae*, 2 – *Junco-Molinietum caeruleae succiselletosum inflexae* 3 – *Junco-Molinietum caeruleae typicum*, 4 – *Selino-Molinietum caeruleae*, 5 – *Carici davallianae-Molinietum caeruleae*.
Tabela 1: Skrajšana sinoptična tabela (prisotnost v %) preučevanih rastlinskih združb mokrotnih travnikov z vrsto *Molinia caerulea*:

relevé number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
relevé code	163	170	162	164	168	169	165	166	178	173	174	175	208	209	210	180	181	171	172	147	148
year	04	04	04	04	04	04	04	04	04	04	04	04	04	04	04	04	04	04	04	04	04
month	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8	8	8	8	6	6
day	1	29	1	1	6	29	1	1	12	3	3	3	20	24	24	12	12	3	3	11	11
plot size (m2)	15	0	24	18	20	24	18	18	24	20	20	24	20	18	20	20	20	24	20	20	18
altitude (m)	520	520	520	520	520	520	520	520	482	482	482	482	425	425	425	483	485	490	485	240	240
number of taxa	29	42	29	43	46	54	69	61	31	30	21	14	17	16	15	39	45	37	42	29	30
characteristic and differential taxa of the association *Plantagini altissimae-Molinietum caeruleae*																					
Sanguisorba officinalis	+	1	3	3	3	2	2	2	.	.	.	.	.	.	.	.	.	.	.	.	.
Plantago altissima	3	3	3	+	2	4	+	+	+	+	.	.	.	.	.	r	.	.	.	.	.
Gratiola officinalis	+	1	2	+	3	3	+	1	3	3	+	+	.	.	.	1	2	.	.	.	.
Centaurea carniolica	+	+	1	+	1	1	2	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Cirsium rivulare	.	.	+	+	+	+	1	+	+	.	.	.	.	.	.	.	1	.	1	.	.
Serratula tinctoria	+	+	+	+	+	+	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.
Ranunculus flammula	2	4	+	.	+	+	.	.	.	3	.	.	.	.	.	+	.	.	.	.	1
Carex nigra	+	1	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	+
characteristic species of the association *Junco-Molinietum caeruleae*																					
Juncus conglomeratus	+	3	1	+	+	3	1	1	+	+	+	.	.	.	.	+	+	2	3	.	.
Juncus effusus	.	+	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	+	+	1
differential species of the subassociation *succiselletosum inflexae* subass. nova																					
Succisella inflexa	4	.	.	.	.	.	+	.	4	3	3	1	3	2	3	3	.	.	.	.	.
characteristic species of the association *Selino-Molinietum caeruleae*																					
Selinum carvifolia	.	.	1	1	1	1	2	+	.	.	.	.	.	.	.	.	.	.	3	.	.
characteristic and differential species of the association *Carici davallianae-Molinietum caeruleae*																					
Carex hostiana	.	+	.	.	.	.	.	.	1	.	.	.	+	+	.	2	+	.	.	.	.
Epipactis palustris	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.
Molinion																					
Molinia caerulea	1	2	4	5	5	3	4	3	4	4	5	5	4	5	5	4	4	4	4	5	4
Succisa pratensis	.	+	.	+	+	+	1	+	+	.	.	.	.	.	.	.	1	+	1	.	.
Gentiana pneumonanthe	+	.	1	.	+	.	.	.	+	+	+	+	.	.	.	+	+	+	+	.	.
Galium boreale	.	.	+	.	3	+	+	.	.	.	.	.	.	.	.	.	.	+	.	.	.
Carex distans	.	.	.	+	.	.	.	.	+	.	.	.	.	.	.	+	.	.	.	.	.
Molinia arundinacea	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	+	.	.
Inula salicina	.	.	.	.	.	.	.	.	.	.	.	.	2	+	.	+	.	.	.	.	.
Iris sibirica	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ophioglossum vulgatum	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Vicia tetrasperma	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.
Molinietalia																					
Juncus acutiflorus	3	2	.	+	2	2	+	1	+	+	1	.	.	.	.	+	3	3	2	.	.
Valeriana dioica	+	1	1	+	+	1	.	.	2	.	+	.	.	.	.	2	2	1	.	.	+
Filipendula ulmaria	+	.	+	2	1	1	+	+	+	+	1	2	.	+	+	+	+	+	2	+	+
Betonica officinalis	.	+	+	1	+	+	3	3	.	.	.	.	.	.	.	.	+	.	+	.	.
Lythrum salicaria	1	+	.	+	.	1	+	.	1	+	+	1	+	+	1	+	+	2	1	+	1
Equisetum palustre	.	+	.	.	+	1	.	.	.	+	+	1	1	3	2	+	.	.	.	+	2
Lysimachia vulgaris	.	1	.	+	.	+	.	.	+	+	1	1	1	2	2	+	.	+	+	2	+
Angelica sylvestris	.	.	.	.	.	+	+	+	.	.	.	.	.	.	.	.	.	.	1	.	.
Genista tinctoria	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.
Linum catharticum	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lychnis flos-cuculi	+	+	.	+	+	+	+	+	.	.	.	.	.	.	.	.	.	+	+	+	.
Myosotis scorpioides	+	1	+	+	+	+	+	.	.	+	.	.	.	.	.	.	.	.	.	.	.
Deschampsia cespitosa	.	.	.	.	.	1	+	1	.	.	.	.	.	.	.	.	+	.	.	+	.
Thalictrum lucidum	.	.	.	.	.	.	.	.	+	.	+	+	+	+	+	+	+	.	.	.	.
Lotus pedunculatus	.	.	.	.	.	.	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.
Senecio aquaticus agg.	1	+	.	+	+	.	.	.	+	2	.	.	.	.	.	1	.	.	.	.	.
Cardamine pratensis agg.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.
Peucedanum coriaceum	.	.	1	.	+	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Crepis paludosa	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Dactylorhiza maculata	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Colchicum autumnale	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Hypericum tetrapterum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.
Molinio-Arrhenatheretea																					
Ranunculus acris	1	+	1	1	1	1	1	2	1	+	+	.	.	.	.	2	2	+	+	1	2
Prunella vulgaris	1	+	.	1	+	3	3	1	2	3	.	.	+	+	.	1	+	.	.	+	.
Lotus corniculatus	+	.	.	+	1	+	+	+	+	.	.	.	+	.	.	+	1	.	.	.	.
Leontodon danubialis	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	+	2	.	.	.	.
Centaurea jacea	+	.	+	.	+	.	1	+	.	.	.	.	+	.	.	.	+	.	.	.	.
Centaurea macroptilon	.	.	.	.	+	.	+	.	.	.	.	.	.	.	.	+	+	.	.	.	+
Holcus lanatus	+	+	.	+	+	1	1	2	.	.	.	.	.	.	.	.	+	+	+	2	2
Lathyrus pratensis	.	.	.	2	+	1	1	2	r	.	.	.	.	.	.	.	+	+	1	.	.
Festuca pratensis	.	+	.	+	.	+	2	2	.	+	.	.	.	.	.	.	+	.	.	+	+
Ajuga reptans	.	+	.	.	.	+	1	1	.	.	.	.	.	.	.	.	.	2	1	+	.
Dactylis glomerata	.	.	.	.	.	+	+	1	.	.	.	.	.	.	.	.	.	.	.	.	.
Leucanthemum ircutianum	.	.	+	.	+	1	1	+	.	.	.	.	.	.	.	.	.	.	.	.	+
Plantago lanceolata	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	+	.	.	.	+
Ranunculus repens	2	3	.	2	.	+	3	+	2	2	+	.	.	.	.	+	+	r	+	.	+
Trifolium pratense	.	.	.	+	.	1	+	+	.	.	.	.	.	.	.	.	r	.	.	.	.
Anthoxanthum odoratum	.	+	.	.	.	+	+	+	.	.	.	.	.	.	.	.	.	.	+	2	2
Vicia cracca	.	.	.	+	.	.	+	+	.	.	.	.	.	+	.	.	.	1	+	.	.
Poa pratensis	.	.	.	.	.	+	+	+	.	.	.	.	.	.	.	.	.	+	+	.	.
Rumex acetosa	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	r	+	+	+
Agrostis stolonifera agg.	.	+	.	.	.	+	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.
Mentha x verticillata	.	.	.	.	.	.	.	.	1	+	+	.	.	.	.	+	+	.	.	.	.
Galium mollugo agg.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	+	1	1	.
Cynosurus cristatus	.	.	.	.	.	.	1	+	.	.	.	.	.	.	.	.	.	.	.	.	+
Taraxacum officinale agg.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.

Trifolium repens	.	+	.	1	+	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Trifolium dubium	.	+	.	.	+	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Festuca rubra agg.	.	.	.	.	.	+	+	1	.	.	.	.	.	.	.	.	+	.	.	.	.	.
Cerastium holosteoides	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	2	+	
Festuca nigrescens	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	
Carex hirta	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	
Achillea millefolium	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	
Trifolium patens	.	.	.	+	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	
Plantago major	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Poa trivialis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	1	
Lotus tenuis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	
Leucanthemum praecox	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Alopecurus pratensis	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Avenula pubescens	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Stellaria graminea	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Scheuchzerio-Caricetea fuscae																						
Carex panicea	4	4	3	1	1	3	2	1	3	4	2	1	4	3	3	4	3	3	2	+	1	
Parnassia palustris	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	+	.	.	.	.	.	
Juncus articulatus	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Agrostis canina	.	+	+	.	+	.	1	+	.	+	.	.	.	.	.	.	.	+	.	.	.	
Carex flava	.	.	+	+	.	.	+	+	.	+	.	.	.	.	.	.	.	.	.	.	+	
Taraxacum palustre	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	
Dactylorhiza incarnata	.	.	+	.	+	+	.	+	.	.	.	.	.	.	.	+	+	.	.	.	.	
Carex lepidocarpa	.	.	.	.	+	.	.	.	.	+	.	.	.	.	.	.	.	+	.	.	+	
Orchis palustris	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	1	
Festuco-Brometea																						
Galium verum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	
Briza media	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	+	.	.	.	.	
Filipendula vulgaris	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	
Allium carinatum	+	.	1	+	1	1	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	
Carex flacca	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Brachypodium pinnatum agg.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	
Trifolium montanum	.	.	.	.	.	.	+	1	.	.	.	.	.	.	.	.	.	.	.	.	.	
Bromus erectus	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	
Trifolium ochroleucon	.	.	.	.	+	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Calluno-Ulicetea																						
Potentilla erecta	+	+	3	2	3	2	1	2	1	+	1	+	1	.	.	1	2	1	3	3	1	
Danthonia decumbens	.	+	.	.	.	+	1	+	.	.	.	.	.	.	.	+	+	.	.	.	.	
Festuca filiformis	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	+	.	.	.	.	
Carex pallescens	.	.	.	+	.	.	+	+	.	.	.	.	.	.	.	.	.	.	+	.	.	
Thymus pulegioides	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	
Hieracium umbellatum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	+	.	.	
Luzula campestris agg.	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	
Calluna vulgaris	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	
Viola canina	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	
Nardus stricta	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	
Rhinanthus glacialis	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	
Cuscuta epithymum	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Phragmito-Magnocaricetea																						
Galium palustre	+	1	+	1	+	+	.	+	+	+	+	+	2	1	1	1	+	+	+	+	1	
Carex acutiformis	.	1	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	
Mentha aquatica	+	1	+	+	+	+	.	.	.	.	.	+	+	+	1	1	.	.	.	1	1	
Carex acuta	.	.	.	+	.	.	+	+	.	.	.	.	.	.	+	.	.	.	.	1	2	
Lycopus europaeus	.	+	.	.	.	+	+	.	+	.	1	+	.	.	.	.	.	.	.	.	.	
Phragmites australis	.	.	.	.	.	.	.	.	.	.	.	.	1	1	2	.	.	.	.	.	.	
Carex elata	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Iris pseudacorus	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	
Carex otrubae	.	.	.	+	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	
Scutellaria galericulata	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	+	.	.	.	
Late-successional taxa																						
Veronica chamaedrys	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	+	1	.	.	
Frangula alnus	.	.	.	.	.	.	.	.	r	.	+	.	.	.	.	r	r	+	+	.	.	
Cruciata glabra	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	+	.	.	
Alnus glutinosa	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	r	.	.	.	.	
Quercus robur	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	.	.	
Solidago gigantea	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	
Salix cinerea	.	.	.	.	.	+	.	.	.	+	.	.	r	.	+	.	r	.	.	.	.	
Salix repens / rosmarinifolia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	+	.	.	
Anemone nemorosa	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Epilobium parviflorum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	
Veronica officinalis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	
Viburnum opulus	.	.	.	.	.	.	.	.	r	.	.	.	.	.	.	.	.	1	.	.	.	
Lathyrus linifolius	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	
Tilia cordata	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	.	.	
Euonymus europaeus	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	
Other taxa																						
Equisetum arvense	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	
Erigeron annuus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	
Elymus repens	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	
Calamagrostis epigejos	.	.	.	.	.	+	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	
Rubus sp.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	
Sisyrinchium bermudiana agg.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	3	+	.	.	.	.	
Equisetum ramosissimum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	2	

Table 2: Analytical table of the plant associations: (1) – *Plantagini altissimae-Molinietum caeruleae*, (2) – *Junco-Molinietum caeruleae succiselletosum inflexae*, (3) – *Junco-Molinietum caeruleae typicum*.

Tabela 2: Analitična tabela asociacij: (1) – *Plantagini altissimae-Molinietum caeruleae*, (2) – *Junco-Molinietum caeruleae succiselletosum inflexae*, (3) – *Junco-Molinietum caeruleae typicum*.

relevé number	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59
relevé code	106	107	179	91	199	23	111	7	214	198	112	113	72	96	97	98	101	135	27	133	136	137	25	26	28	29	30	31	32	134	8	67	68	69	70	71	74	75
year	03	03	04	03	04	03	03	03	03	04	04	03	03	03	03	03	03	04	03	04	04	04	04	03	03	03	03	03	03	04	03	03	03	03	03	03	03	03
month	8	8	8	7	8	8	7	8	8	8	7	7	6	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	6	6	6	6	6	6	6	6
day	18	18	12	2	20	21	2	25	5	20	2	2	30	12	12	13	13	5	21	5	5	5	21	21	21	27	27	27	27	5	27	30	30	30	30	30	30	30
plot size (m2)	18	24	24	24	20	18	20	15	18	20	20	24	24	20	20	18	16	25	20	20	24	24	16	18	20	16	16	20	20	20	24	24	20	24	20	24	20	30
altitude (m)	322	322	483	323	535	685	323	350	490	540	322	322	436	323	323	323	323	530	684	530	530	530	680	678	680	680	678	685	685	530	265	437	436	436	436	436	435	435
number of taxa	42	37	42	46	46	37	50	38	61	29	22	32	39	30	31	44	37	26	29	26	23	28	28	29	46	43	34	45	49	29	21	35	35	50	37	34	22	21
characteristic and differential taxa of the association *Plantagini altissimae-Molinietum caeruleae*																																						
Plantago altissima	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gratiola officinalis	3	4	1	3	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Centaurea carniolica	.	.	.	+	.	.	+	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	+	+	+	.	.	.	.	.	.	.	.	+	.	.	.	.
Cirsium rivulare	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Serratula tinctoria	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.
Ranunculus flammula	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
characteristic species of the association *Junco-Molinietum caeruleae*																																						
Juncus conglomeratus	+	+	+	.	.	1	.	.	.	.	.	+	.	.	+	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.
Juncus effusus	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
differential species of the subassociation *succiselletosum inflexae* subass. nova																																						
Succisella inflexa	+	+	.	+	.	.	.	.	.	.	.	+	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
characteristic species of the association *Selino-Molinietum caeruleae*																																						
Selinum carvifolia	+	+	3	+	.	+	+	2	3	.	.	+	.	3	3	2	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Laserpitium prutenicum	+	+	.	.	r	.	.	+	1	2	+	+	.	3	2	2	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
characteristic and differential species of the association *Carici davallianae-Molinietum caeruleae*																																						
Carex davalliana	.	.	.	.	.	.	.	1	1	.	.	.	.	.	.	.	.	4	2	3	3	3	+	+	3	2	1	2	1	1	+	2	.	+	1	3	5	5
Carex hostiana	+	+	1	+	.	.	1	+	.	1	2	1	1	.	+	+	.	1	3	3	2	2	.	+	+	1	.	2	1	1	4	3	4	2	2	3	1	2
Epipactis palustris	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	2	+	1	+	1	+	+	2	2	.	+	1	r	.	+	+	+	+	1	1	1
Koeleria pyramidata	.	.	.	.	.	+	.	+	.	.	.	.	1	.	.	+	.	.	+	+	.	.	+	+	+	+	.	+	.	+	+	2	1	2	1	1	+	+
Eriophorum latifolium	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	+	+	.	+	+	.	.	.	+	+	+	+	.	.	+	.	.	.	1	.	+
Molinion																																						
Molinia caerulea	4	5	4	3	4	5	4	5	4	4	5	5	5	4	4	4	4	4	4	4	4	4	5	4	5	5	4	4	5	5	4	4	4	5	5	5	4	5
Succisa pratensis	1	+	1	4	+	1	4	2	1	+	+	1	+	2	1	.	+	2	2	3	3	3	+	2	+	1	+	.	+	1	1	+	+	+	+	1	+	+
Gentiana pneumonanthe	+	+	1	+	.	.	+	.	.	.	1	+	.	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Galium boreale	+	2	+	.	2	.	.	.	+	3	.	+	+	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3	3	2	+	+	.
Carex distans	.	.	+	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	.	.	1	.	+	+	.	.	.
Molinia arundinacea	.	.	.	.	+	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	+	.	.	.	.
Inula salicina	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.
Iris sibirica	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gladiolus palustris	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	1	.	.	.	.
Carex tomentosa	.	.	+	.	.	.	.	.	.	.	.	.	.	+	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gentiana asclepiadea	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.
Polygala amarella	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Molinietalia																																						
Valeriana dioica	1	.	1	.	2	1	.	+	1	.	.	.	.	.	.	+	.	1	1	1	3	+	+	1	1	2	3	1	2	+	.	.	.	.	.	.	+	.
Filipendula ulmaria	+	+	2	+	+	.	.	.	.	+	+	.	.	.	.	+	+	r	.	.	.	+	+	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.
Betonica officinalis	+	+	.	1	.	.	2	.	.	2	1	2	2	3	3	4	2	.	.	.	.	.	.	.	+	.	.	+	.	.	+	1	1	3	2	+	+	+
Lythrum salicaria	.	+	+	.	+	+	.	.	+	.	.	.	.	.	.	.	.	+	+	.	.	.	.	+	.	+	.	.	+	.	.	+	.	.	.	.	+	.
Equisetum palustre	.	.	.	.	.	1	.	2	3	.	.	.	.	.	.	.	.	+	2	2	4	3	2	2	+	2	3	+	3	+	.	.	.	.	.	.	.	.
Lysimachia vulgaris	+	+	+	.	r	.	.	.	.	.	.	+	.	.	.	+	.	+	.	.	.	r	.	.	.	.	+	.	+	.	.	1	.	.	.	.	.	.
Angelica sylvestris	+	+	+	.	.	1	.	.	.	.	+	+	.	.	.	.	.	r	+	+	+	.	1	+	+	+	+	1	+	1	.	.	.	.	.	.	.	.
Genista tinctoria	.	.	.	1	.	.	2	.	.	+	.	1	1	1	1	1	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	2	+	1	1	.	+
Linum catharticum	.	.	.	+	.	.	+	.	+	+	.	.	.	+	.	.	.	.	+	+	.	.	.	+	+	+	.	+	.	.	.	+	.	.	.	.	.	.
Cirsium oleraceum	.	.	.	.	2	2	.	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	+	+	+	1	.	+	+
Gymnadenia conopsea	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	+	+	.	+	+	1	+	+	.	+	+	.	.	.	.	+	+	.	.	.
Lychnis flos-cuculi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Myosotis scorpioides	.	.	.	.	.	+	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Deschampsia cespitosa	.	.	.	1	+	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.
Thalictrum lucidum	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lotus pedunculatus	.	.	.	.	+	.	.	.	.	+	.	.	.	.	.	.	.	.	.	+	.	+	.	.	.	.	.	.	.	.	.	+	.	+	.	+	.	.
Cirsium palustre	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	+	.	.	.	.	+	+	+	.	+	.	.	.	.	.	.	.	.
Geum rivale	.	.	.	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	+	+	+	+	.	.	.	.	.	.	.	.	.
Juncus acutiflorus	+	+	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cardamine pratensis agg.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Galium uliginosum	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	+	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Crepis paludosa	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Dactylorhiza maculata	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Colchicum autumnale	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Veratrum album	.	.	.	.	.	.	.	.	.	1	.	.	+	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hypericum tetrapterum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.
Scirpus sylvaticus	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Allium angulosum	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Caltha palustris	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Molinio-Arrhenatheretea																																						
Ranunculus acris	+	+	+	1	2	1	1	+	1	1	+	+	1	1	+	+	+	+	2	2	.	2	3	4	2	2	3	1	1	1	+	2	2	1	1	1	+	1
Prunella vulgaris	.	.	+	1	+	.	1	.	2	.	.	.	1	.	.	.	+	+	+	+	.	r	+	+	1	.	.	+	+	+	.	1	1	.	+	+	.	.
Lotus corniculatus	+	+	+	1	.	.	1	.	+	.	.	+	2	+	+	.	+	.	+	.	.	.	1	.	+	+	.	+	1	.	+	2	1	1	1	1	+	.
Leontodon danubialis	.	+	+	1	+	.	+	.	1	1	.	.	2	.	.	.	.	+	+	+	+	2	.	.	+	+	1	+	+	+	1	3	2	1	3	2	.	+
Centaurea jacea	1	+	+	2	+	+	1	+	+	r	+	+	.	.	+	1	+	.	.	.	.	.	.	.	+	+	.	+	+	.	.	.	.	.	.	.	.	.
Centaurea macroptilon	1	+	+	2	.	.	2	.	1	.	1	1	.	.	+	+	+	.	.	+	.	.	.	.	+	+	.	+	+	+	+	.	.	.	.	.	.	.
Holcus lanatus	+	+	.	1	+	+	1	+	.	.	+	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.
Lathyrus pratensis	.	.	+	.	1	2	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	+	1	+	.	2	+	+	+	.	.	.	.	.	.	.	.
Festuca pratensis	.	.	.	1	+	+	+	.	+	.	+	.	.	.	.	.	.	.	.	+	.	.	.	.	+	.	.	+	+	+	.	.	.	.	.	.	.	.
Ajuga reptans	+	+	.	.	+	.	.	.	+	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	+	+	+	1	+	.	.	+	+	.	.	.	.	.
Dactylis glomerata	.	.	.	.	+	.	+	.	+	+	.	.	.	.	+	.	+	.	.	.	.	.	.	.	+	+	.	+	+	.	+	1	+	1	1	+	.	.
Leucanthemum ircutianum	.	.	.	+	.	.	.	.	.	.	.	.	+	.	.	.	.	.	+	.	.	+	+	+	+	+	+	+	+	.	.	.	.	+	.	+	.	.

Plantago lanceolata	+	.	.	.	.	.	+	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	+	.	+	+	.	.	+	.	+	+	+	+	+	+	+
Ranunculus repens	+	.	+	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Trifolium pratense	.	.	.	+	+	.	+	.	+	.	.	.	+	.	.	.	.	.	.	.	.	.	.	+	+	.	.	+	.	.	.	.	.	.	.	.	.	.
Anthoxanthum odoratum	.	.	.	+	.	.	1	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	+	.	.	.	.	.	.	.	.
Vicia cracca	.	.	.	.	+	+	.	+	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.
Poa pratensis	.	.	+	.	.	+	.	.	+	.	.	.	.	+	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rumex acetosa	.	.	.	.	.	+	.	.	.	+	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.
Agrostis stolonifera agg.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	+	.	.	.
Mentha x verticillata	1	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Festuca rubra	.	+	.	1	.	.	2	.	.	.	.	1	.	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Galium mollugo agg.	.	.	.	.	.	+	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3	.	.	.	.	.	.	.	.
Cynosurus cristatus	+	+	.	+	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Leontodon hispidus	.	.	.	+	.	.	+	.	+	.	.	.	.	.	.	.	.	.	.	+	+	+	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.
Taraxacum officinale agg.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.
Trifolium dubium	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Festuca nigrescens	.	.	.	+	.	.	+	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Carex hirta	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.
Festuca arundinacea	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	+	.	.	.	.
Galium album	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	+	+	.	1	.	.	.	.	.	.	.	.	.	.
Euphrasia rostkoviana	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	+	+	.	.	.	.	.	.	.	.	.	.
Achillea millefolium	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Juncus inflexus	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	+	.	.	.	.
Pimpinella major	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.
Rhinanthus minor	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.
Daucus carota	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Centaurea jacea agg.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.
Arrhenatherum elatius	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Potentilla reptans	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Tragopogon orientalis	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Scheuchzerio-Caricetea fuscae																																						
Carex panicea	2	1	2	+	3	.	1	1	3	2	1	.	+	1	+	+	.	3	1	2	3	3	1	1	1	2	2	2	1	2	+	.	.	+	+	1	+	+
Parnassia palustris	.	.	+	.	+	.	.	+	.	+	.	.	.	.	.	.	.	2	2	2	2	3	+	+	+	1	+	2	1	1	.	.	.	.	.	.	+	+
Juncus articulatus	.	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	+	+	+	+	+	.	.	.	+	+	.	+	.	3	+	.	.	.	.	.	.
Agrostis canina	1	2	.	+	.	.	1	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Carex flava	+	.	+	.	.	1	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.
Taraxacum palustre	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	1	+	1	+
Carex pulicaris	+	.	.	2	.	.	1	.	.	.	2	2	.	+	1	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Carex lepidocarpa	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Orchis palustris	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	+	.
Juncus alpinoarticulatus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	1	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Menyanthes trifoliata	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	3	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Epilobium palustre	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.
Carex echinata	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Tofieldia calyculata	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Schoenus nigricans	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Primula farinosa	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Festuco-Brometea																																						
Galium verum	+	.	+	+	+	.	2	.	+	1	.	+	2	1	2	3	1	.	.	.	.	.	.	.	1	+	.	1	.	.	.	1	2	2	.	.	.	.
Briza media	.	.	.	1	.	+	2	.	+	.	.	+	.	+	+	+	+	+	+	1	+	+	.	+	+	+	+	+	+	1	+	1	+	+	+	+	.	.
Filipendula vulgaris	.	.	.	1	.	.	1	.	.	.	.	.	2	1	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	1	+	2	1	+	+	.	.
Allium carinatum	.	.	+	+	.	.	+	.	+	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	+	+	1	+	+	+	.	+
Centaurea pannonica	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	2	+	.	.	.
Carex flacca	.	.	.	+	.	.	.	.	.	.	.	.	1	+	+	.	1	.	.	.	.	.	.	.	.	+	.	+	.	.	+	.	+	1	+	+	+	+
Pimpinella saxifraga	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Brachypodium pinnatum agg.	.	.	.	.	.	.	+	.	1	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	.	.	+	+	.	+	+	1	+	+	.	.
Trifolium montanum	.	.	.	+	.	.	+	.	+	+	+	+	+	+	+	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	+	+	.	.	.
Campanula glomerata	.	.	.	+	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.
Sedum sexangulare	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Plantago media	.	.	.	.	r	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	+	+	.	.	.	.	.	+	.	.	+	+	.	.
Prunella grandiflora	.	.	.	.	.	.	.	.	+	2	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3	+	+	.	.
Gentiana verna	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	.	.	+	.	.	.	.	.	.	.	.	.	.
Ononis spinosa	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	+	.	.	.
Buphthalmum salicifolium	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.
Danthonia alpina	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Calluno-Ulicetea																																						
Potentilla erecta	2	.	3	3	3	1	2	3	3	3	2	2	2	3	3	4	3	1	3	3	3	3	3	3	3	3	1	3	3	3	2	3	3	2	2	3	2	3
Danthonia decumbens	+	+	.	.	.	.	3	.	.	+	.	+	1	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	+	+	.	.	.	.
Festuca filiformis	+	.	+	1	.	.	1	.	.	.	.	2	+	1	1	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	+	.	.
Carex pallescens	.	.	+	+	.	+	+	+	.	.	.	.	.	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Thymus pulegioides	.	.	.	.	+	.	.	.	+	+	.	.	+	.	.	+	.	.	.	.	.	.	.	.	.	+	.	+	+	.	.	.	.	+	.	.	.	.
Hieracium umbellatum	+	+	.	.	.	.	.	.	.	.	.	+	.	1	+	+	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Luzula campestris agg.	+	+	.	+	.	.	+	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Calluna vulgaris	+	.	.	.	.	.	+	.	.	.	.	+	.	+	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Viola canina	.	.	2	+	.	.	+	.	.	.	.	.	+	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.
Nardus stricta	+	.	.	2	.	.	2	.	.	.	.	.	.	.	+	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Polygala vulgaris	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.
Hieracium lactucella	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	+	.	.	.	.	.	.	.	.	.	.	.
Rhinanthus glacialis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.
Agrostis capillaris	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Phragmito-Magnocaricetea																																						
Galium palustre	+	+	+	.	.	+	.	+	.	.	+	.	.	+	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mentha aquatica	.	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	1	.	.	2	.	.	.	.	+	.	.	1	.	.	.	.	.	+	+	.	1
Carex acuta	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.
Lycopus europaeus	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Carex vesicaria	.	.	.	.	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Carex vulpina	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Phragmites australis	.	.	.	.	.	.	.	2	.	+	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	1	1	2	1	+	2
Peucedanum palustre	.	.	.	.	.	.	.	.	.	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	.	.
Carex elata	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.

Iris pseudacorus	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Carex paniculata	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.
Late-successional taxa																																						
Veronica chamaedrys	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.
Frangula alnus	+	+	+	.	.	.	.	+	r	.	+	+	.	.	.	.	+	.	+	.	.	.	.	.	+	.	.	.	+	.	.	.	.	.	.	.	.	.
Cruciata glabra	.	.	.	.	+	+	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	1	+	+	.	.	.	.	.	.	.	.	.
Alnus glutinosa	.	.	+	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	1	+	+	+	+	+	+	.	.	.	.	.	.	.	+	.
Quercus robur	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	r	.	.
Knautia drymeia	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	+	.	.	.	.	.	+	.	+	+	1	+	1	.	.	.	.	.	.	.	.	.
Salix cinerea	.	.	+	.	1	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Salix repens / rosmarinifolia	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	3	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.
Eupatorium cannabinum	.	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	+	+	.	+	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.
Fraxinus excelsior	.	.	.	.	r	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	r	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Epilobium parviflorum	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Pinus sylvestris	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gymnadenia odoratissima	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	.	.	.	+	.	.	.	.	+	.	.	.	.	.
Viburnum opulus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.
Phyteuma spicatum	.	.	.	.	+	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Laserpitium latifolium	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Calamagrostis arundinacea	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.
Asarum europaeum	.	.	.	.	.	.	.	.	r	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Carpinus betulus	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lathyrus linifolius	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Primula vulgaris	.	.	.	.	+	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Erica carnea	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.
Lathyrus latifolius	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cornus sanguinea	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Solidago virgaurea	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Other taxa																																						
Equisetum arvense	.	.	.	.	+	.	+	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.
Chaerophyllum sp.	.	.	.	.	.	+	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Scabiosa triandra	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	+	.	+	1	.	+	.	.	.	.	.	+	.	.	.	.
Betula pendula	.	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Picea abies	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	+	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.
Populus tremula	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Salix purpurea	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Corylus avellana	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.
Prunella sp.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.
Orobanche sp.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table 3: Analytical comparable table of the plant associations: (4) – *Selino-Molinietum caeruleae*, (5) – *Carici davallianae-Molinietum caeruleae*.

Tabela 3: Analitična tabela asociacij: (4) – *Selino-Molinietum caeruleae*, (5) – *Carici davallianae-Molinietum caeruleae*.

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Status and distribution of the lynx (*Lynx lynx*) in the Swiss Alps 2005–2009

Status in razširjenost risa (*Lynx lynx*) v Švicarskih Alpah 2005–2009

[*a]Fridolin Zimmermann, [a]Anja Molinari-Jobin, [a]Andreas Ryser, [a]Christine Breitenmoser-Würsten, [a]Elias Pesenti, [b]Urs Breitenmoser

[a]KORA,Thunstrasse 31, CH-3074 Muri, Switzerland Thunstrasse 31, CH-3074 Muri, Switzerland
[b]Institute of Veterinary Virology, University of Bern, Länggassstrasse 122, CH-3012 Bern, Switzerland
[*]correspondence: f.zimmermann@kora.ch

Abstract: We evaluated the status of lynx in the Swiss Alps for the period 2005–2009. Even though the number of lynx presence signs remained almost stable between the present (2,068 signs) and previous pentad (2,091), there was a 7.6% increase in the area occupied by the 5-km circular buffers around the confirmed lynx signs of presence over the five years period (12,637 km^2). The north-western Swiss Alps (VI) remained the compartment with the highest number of chance observations. It was followed by compartments central Switzerland west (III) and north-eastern Switzerland (II). These sub-populations acted as source in the current pentad, as signs of reproduction were reported almost every year. The translocation to north-eastern Switzerland is still the only significant contribution to the spatial increase of the lynx range in the last 10 years in the Swiss Alps. The small and vulnerable north-eastern Switzerland lynx sub-population plays an important role for the Alpine population. There is hope that in the future this sub-population could act as stepping stone to the eastern Alps and together with individuals dispersing from the central Switzerland west (III) sub-population would enable to found a new sub-population in central Switzerland east (IV). The status of the sub-population in the Valais (VII) is less clear. As only few signs of reproduction and mortalities were reported over the pentad, it acted more as sink than a source population. From the few signs of lynx presence reported in the remaining compartments (Grisons V, central Switzerland east IV and Ticino VIII) we concluded that only a few single lynx that did not yet establish the typical social organisation occur there. An occupancy-based population estimate from a parallel study resulted in about 111 (SE = 10) independent lynx for the period 2005–2009. This is higher than the 60–90 individuals estimated for the previous pentad.

Keywords: Alps, distribution, *Lynx lynx*, monitoring, status, Switzerland

Izvleček: Prispevek ocenjuje stanje risa v Švicarskih alpah za obdobje 2005–2009. Čeprav je številčnost znakov prisotnosti risa med sedanjim (2068) in prejšnjim (2091) pet-letnim obdobjem ostala stabilna, se je območje skupaj s 5-km pufersko cono povečalo za 7,6 %. SZ Švicarske alpe (IV) tako ostajajo območje z najvišjim številom opažanj. Sledita območji osrednje Z Švice (III) in SV Švice (II). Omenjene sub-populacije so bile vir opažanj za zadnje pet-letno obdobje, saj so bili znaki reprodukcije prisotni skoraj vsako leto. Širjenje na območje SV Švice je edino prostorsko

povečanje areala v Švicarskih alpah v zadnjih 10 letih. Majhne in ranljive SV švicarske sub-populacije risa imajo pomembno vlogo za vzdrževanje risa v Švicarskih alpah. Ostaja upanje, da bo ta populacija odigrala vlogo odskočne deske do V Alp in skupaj s posameznimi osebki, ki prihajajo iz osrednje švicarske »sub-populacije (III)« in bo omogočila nastanek nove sub-populacije v osrednji V Švici (IV). Stanje sub-populacije na območju Valais (VII) je manj jasno. Ker je za zadnje pet-letno obdobje znanih le malo znakov reprodukcije in smrtnosti, predstavlja bolj ponor kot vir. Na podlagi znakov risove prisotnosti na ostalih območjih (Grisons (V), osrednja V Švica (IV) in Ticino (VIII)) smo zaključili, da se tam pojavljajo posamični osebki, ki se še niso povezali v populacijsko strukturo. Po ocenah modela zasedenosti prostora (occupancy models) iz vzporedne študije ocenjujejo 111 (SE = 10) neodvisnih osebkov za obdobje 2005–2009. To je precej več kot 60–90 osebkov ocenjenih za prejšnjo petletko.

Ključne besede: Alpe, razširjenost, *Lynx lynx*, monitoring, status, Švica

Introduction

Two lynx (*Lynx lynx*) populations are currently present in the Alps originating from the reintroduction done in the 1970s. One lies in the western Alps in Switzerland and one in the Slovenian Alps, expanding into Italy and Austria. Switzerland harbours the most vital sub-population (Molinari-Jobin et al. 2010) and thus has a great responsibility regarding the conservation of the Alpine lynx population which has still to be considered as endangered according to the IUCN Red List criteria.

Forty years after the first reintroduction, less than 20% of the whole suitable habitat in the Alps has been recolonized by the species, despite considerable efforts at the national and international level to expand the existing lynx areas. In Switzerland large parts of central Switzerland east, the Grisons, and Ticino are not yet colonized by lynx (Zimmermann et al. 2010a). Lynx experts from the Alps considered illegal killing, habitat fragmentation and anthropogenic accidents to be the main reasons for this slow expansion (Molinari-Jobin et al. 2010).

In Switzerland the main conflict is with hunters who compete with lynx for game, and who fear that high lynx densities diminish ungulate game (Breitenmoser et al. 2010). A side effect of this conflict is that the local authorities become reluctant to actively conserve lynx. A possible solution proposed by hunters' associations and wildlife managers, may be a controlled legal harvest to supress illegal killing. In parallel, this solution might increase the willingness of new cantons to actively reintroduce lynx as this new legislation would give them the rights to intervene under some circumstances. The Swiss Lynx Concept established in 2000 and updated in 2004 defines the general conservation and management goals, the co-operation between the FOEN and the cantons. Besides the removal of stock raiders and the translocation from high density areas to areas not yet colonized to foster the spatial expansion of the lynx population, the Swiss Lynx Concept foresees that lynx are reduced through controlled hunting, if the impact of the lynx predation on roe deer and chamois is considered too strong. However this needs a revision of the hunting ordinance. The consultation of the hunting ordinance ended in October 10th 2011. By mid-2012 the Federal Council will adopt the report of the consultation and the ordinance.

To counterbalance the slow expansion of the lynx population in Switzerland, six lynx were translocated in 2001 from the north-western Alps to north-eastern Switzerland. Another three from the Jura Mts. followed in 2003. All animals were fit with radio-collars in order to follow their movements, reproduction events and mortalities. In Switzerland the translocation of a total of 6 lynx to the north-east in 2001–2003 (Ryser et al. 2004) led to an increase of 7% of the lynx distribution range in the whole Alps (Molinari-Jobin et al. 2010). The monitoring conducted in winter 2005/06 in north-eastern Switzerland revealed that the lynx number was critically low (Ryser et al. 2006). Subsequently, in 2006 the north-eastern Swiss cantons and the FOEN based on recommendations

from the program KORA decided to restock the north-eastern Switzerland lynx sub-population with additional 3–4 individuals that should mainly originate from the Jura Mts. to increase the chance of mixing up the genes of both meta-populations (Jura Mts. and Alps). In 2007, one male and one female lynx were translocated from the Jura Mts. and north-western Swiss Alps, respectively. Another female was translocated in 2008 from the Jura Mts. (KORA unpublished data). Similarly to the 2001 and 2003 translocations all individuals were fit with radio-collars to monitor the fate of individuals during the first years after their release. The translocation project ended in 2009 and was since then integrated into the national monitoring.

The purpose of this report is to evaluate the status of lynx in the Swiss Alps for the period 2005–2009.

Material and methods

For organizational purposes, Switzerland was divided into 8 large carnivore management compartments, taking into account natural and artificial barriers to natural spread of lynx as well as political borders (Fig. 1). We used a stratified approach to monitor the lynx population (Breitenmoser al. 2006) as financial resources are restricted. There is a stratification in space (national level, compartments and smaller reference areas within compartments), in time (e.g. chance observations are gathered year round whereas systematic camera-trapping, which is very labor intensive, is conducted every 2 to 3 years in smaller reference areas) and in the datasets according to the type of observation and their validity (e.g. SCALP criteria; Molinari-Jobin et al. in press). On the national level questionnaires are sent on yearly bases to all game wardens of Switzerland (Capt et al. 1998). These questionnaires provide basic information about the detection/non detection of lynx, mortality, and reproduction as well as a subjective assessment of the trend of the lynx "population" within each game warden's surveillance area over the whole Switzerland. Chance observations (sightings, tracks, wildlife killed) are gathered year round at the national and compartment level. Livestock killed by lynx need to be confirmed by trained people to be compensated, mainly game wardens. All damages to livestock reported are published online on our webpage. This allows an open review when permission for removal of an individual lynx as stockraider is issued by the cantons of the corresponding compartment and the FOEN. Opportunistic camera-trapping, where camera-traps are set on ideal occasions principally at fresh kills, is conducted at the compartment level. At a smaller scale in reference areas (680–1,601 km^2) within three large carnivore compartments (II, III and VI) we estimated the number of lynx using photographic capture-recapture models (e.g. Zimmermann et al. 2010b). These data are reported each year in our national large carnivore monitoring reports (e.g. Zimmermann et al. 2010a) to make this information available to the members of the lynx monitoring network, the decision makers, the NGOs and the general public.

On the national level, five sources of information on the presence of lynx are available: (1) reports of lynx killed or found dead, or young orphaned lynx caught and put into captivity; (2) opportunistic camera trapping where camera-traps are set for ideal occasion, mainly at fresh kills; (3) samples confirmed by means of genetic analysis; (4) records of livestock killed by lynx; and (5) chance observations of wild prey remains, tracks, scats, sightings, and vocalisations. Three levels of reliability were distinguished according to the possibility to verify an observation (Molinari-Jobin et al. in press): Category 1 (C1) represent the hard facts (i.e. direct signs), e.g. all reports of lynx killed, found dead or removed from the wild as young orphaned lynx and put into captivity, as well as opportunistic photographs of lynx. We also include all samples that were identified to be lynx by means of genetic analysis in this category. All lynx photographs of one or more individuals taken at a kill were counted as a single detection. Lynx photographs taken at a given site along a trail were counted as single detection for each night even though several lynx were pictured the same night (this happened only on rare occasions). Category 2 (C2) represent all records of livestock killed, wild prey remains and tracks confirmed by trained people, e.g. mainly game wardens. As all game wardens were instructed how to recognize signs of lynx presence, these records are mostly an objective proof of lynx presence, though both

errors and even deception may occur. Category 3 (C3) represent chance observations of all wild prey remains and tracks reported by the public as well as all sightings, scats and vocalisations, e.g. mainly indirect signs that can hardly be verified. The information about reproduction came from three different data sets: chance observations of juvenile lynx, photographs of juvenile lynx during the opportunistic camera-trapping and juvenile lynx found dead or captured as orphans for removal from the wild.

To be able to compare the spatial range of the lynx population for the pentad 2005–2009 with those reported in previous status reports (Molinari-Jobin et al. 2006), we computed two different measures of the spatial range: (1) the minimum convex polygon (MCP) encompassing all signs of presence belonging to category 2; and (2) a circular buffer of 5-km around the C2 signs of presence, resulting in an area of about 80 km² around each confirmed sign of presence. This area corresponds roughly to an average female lynx home range size in the Alps (Breitenmoser-Würsten et al., 2001).

Results

The number of signs of presence recorded in the Swiss Alps from 2005–2009 (2,068) remained stable compared to the previous pentad (2,091; Molinari-Jobin et al. 2006). Signs of presence (C1–C3) were reported from all compartments, the fewest in the compartment Ticino (VIII) with 8 and the most in the north-western Swiss Alps (VI) with 966 (Fig. 1). Intermediate values were found in the remaining compartments (345 in compartment II, 424 in III, 70 in IV, 68 in V, and 187 in VII).

A total of 155 damages to livestock were reported (Table 1), which is less than one fourth of the number reported for the previous pentad (543). On the other hand, the number of wild prey remains reported almost doubled (801 compared to 449 in the previous pentad).

With 30 the number of lynx found dead or removed from the wild remained almost stable compared to the previous pentad. Most losses occurred in the north-western Swiss Alps (16) and in central Switzerland west (11), followed by north-eastern Switzerland (2) and the Grisons (1).

The signs of reproduction showed almost the same pattern as the reported lynx mortalities (Fig. 2). The largest part of signs of reproduction came from compartments VI (32), II (32) and III (23). The remaining, all unconfirmed signs except one, came from the Valais (VII) with two, from central Switzerland east (IV) with one and from the Grisons with two of which one was a confirmed sign of reproduction, although it came from a juvenile lynx originating from north-eastern Switzerland (II) that died in Grisons during its dispersal.

As in the previous pentad, 71% of the signs of presence belong to the C1 and C2 category and thus have been confirmed. C1 signs of presence considerably increased in north-eastern and central Switzerland west

Category 1	2005	2006	2007	2008	2009	Total
Photo	22	23	41	42	48	176
Dead lynx	3	4	4	9	10	30
Genetic sample		2	1	2	1	6
Total	25	29	46	53	59	212
Category 2						
Livestock killed	31	28	47	21	28	155
Wild prey remains	136	119	148	170	228	801
Tracks	50	54	58	60	70	292
Total	217	201	253	251	326	1,248
Category 3						
Wild prey remains	6	11	15	9	13	54
Tracks	10	18	15	8	5	56
Sightings	82	87	87	97	113	466
Vocalisations	1	3	12	5	2	23
Scats	2	2	1	3	1	9
Total	101	121	130	122	134	608
Total all categories	**343**	**351**	**429**	**426**	**519**	**2,068**

Table 1: Number of lynx records collected per year and category from 2005–2009.

Tabela 1: Število zbranih podatkov o znakih prisotnosti risa po letih in kategoriji v obdobju 2005–2009.

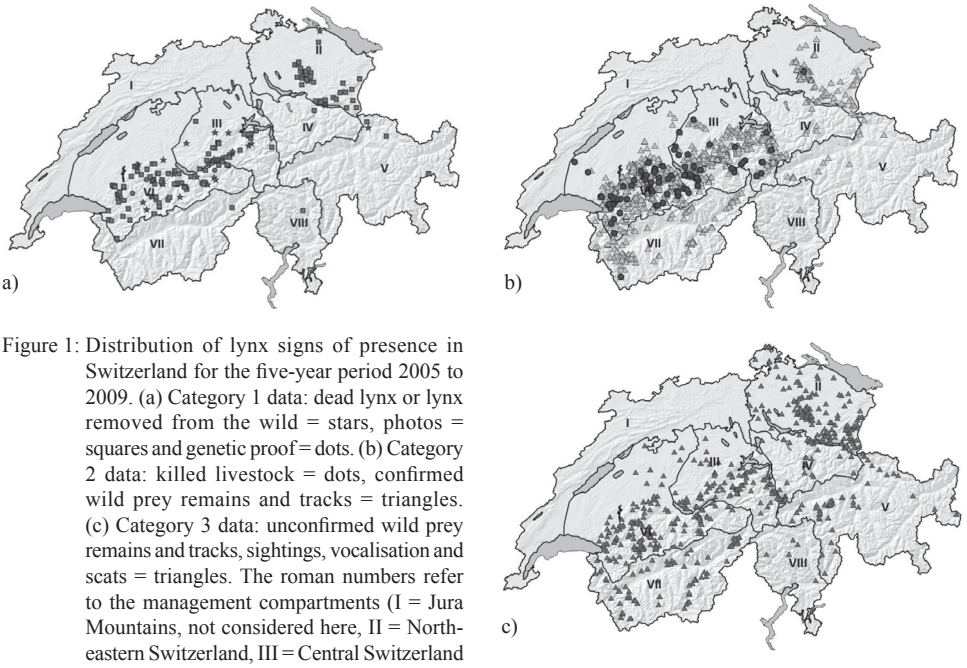

Figure 1: Distribution of lynx signs of presence in Switzerland for the five-year period 2005 to 2009. (a) Category 1 data: dead lynx or lynx removed from the wild = stars, photos = squares and genetic proof = dots. (b) Category 2 data: killed livestock = dots, confirmed wild prey remains and tracks = triangles. (c) Category 3 data: unconfirmed wild prey remains and tracks, sightings, vocalisation and scats = triangles. The roman numbers refer to the management compartments (I = Jura Mountains, not considered here, II = North-eastern Switzerland, III = Central Switzerland west, IV = Central Switzerland east, V = Grisons, VI = North-western Swiss Alps, VII = Valais and VIII = Ticino).

Slika 1: Razporeditev znakov prisotnosti risa v Švici za petletno obdobje 2005–2009. (a) Kategorija podatkov C1: mrtvi risi alirisi odvzeti iz narave = zvezde, fotografije = kvadrati in genetski dokazi = pike. (b) Kategorija podatkov C2: napadi na drobnico = pike, potrjeni ostanki naravnega plena in sledi = trikotniki. (c) Kategorija podatkov C3: ne potrjeni ostanki naravnega plena, sledi, opažanja, oglašanja in iztrebki risa = trikotniki. Rimske številke se nanašajo na upravljalske oddelke (I = Jura, tukaj ni obravnavano, II = severovzhodna Švica, III = osrednja Švica – zahod, IV = osrednja Švica – vzhod, V = Graubünden, VI = severozahodne švicarske Alpe, VII = Valais in VIII = Ticino).

compared to the previous pentad. C1 category data disappeared completely from western Grisons but in parallel some hard facts appeared for the first time in the northern part of the Grisons close to compartment II, where lynx were translocated. Category 2 signs of presence are more sparsely distributed in the western part of the Grisons compared to the previous pentad. For the first time in central Switzerland west (III) some confirmed signs of presence (C2) were recorded South to the Napf region.

The MCP encompassing all C2 signs of lynx presence increased from 20,166 km^2 in 2000–2004 to 27,487 km^2 in 2005–2009. The 5-km buffer around the C2 data resulted in a range estimate of 12,637 km^2 compared to 11,736 km^2 for the previous pentad (2000–2004).

Discussion

Development of lynx signs of presence

About 47% of lynx signs of presence that were reported for this pentad stem from the north-western Swiss Alps (VI) although this compartment contains only about 18% of the suitable lynx habitat in the Swiss Alps (based on 10x10-km cells containing ≥ 10% of suitable habitat fragment > 50 km^2; Zimmermann et al. in prep.). It is followed by compartments central Switzerland west and north-eastern Switzerland with 20.5% and 17%, respectively although the suitably habitat in these compartments makes up only 8% and 11.7% of the suitable lynx habitat, respectively. Each of the remaining compartments

Figure 2: Information about reproduction from three different sources: chance observations = triangles, dead lynx or lynx removed from the wild = stars and opportunistic camera-trapping = squares.

Slika 2: Informacije o reprodukciji iz treh različnih virov: naključna opažanja = trikotniki, mrtvi risi ali risi odvzeti iz narave = zvezde in oportunistični posnetki s foto-pastmi = kvadrati. Table captions.

(IV, V, VII and VIII) contained less than 10% of chance observations that were reported over the five year period although the suitable habitat within these compartments makes up 9% to 25% of the suitable lynx habitat.

In the previous pentad (2000–2004) signs of reproduction were mainly reported in compartments north-western Swiss Alps (VI) followed by central Switzerland west (III) and the Valais (VII) with very few signs. In north-eastern Switzerland (II), where lynx were translocated since 2001, signs of reproduction were only reported for the year 2003. In the current pentad (2005–2009) juvenile lynx were observed and reported every year in compartments north-western Swiss Alps (VI), north-eastern Switzerland (II) and central Switzerland west (III). In the Valais (VII) reproduction was only reported in 2006 and 2007. Even though a juvenile lynx was found dead in the Grisons in 2008, this lynx originated from north-eastern Switzerland (II) as revealed by genetic analyses (Breitenmoser-Würsten 2009). In 2009 for the first time an isolated sighting of a juvenile lynx (C3) was reported in the southern Grisons (V) close to the border with the canton of Ticino (VIII). This needs however to be confirmed in the next pentad. In 2003 compartment central Switzerland east (IV) faced immigration of female AIKA that was translocated to north-eastern Switzerland (Ryser et al. 2004). Even though female AIKA was still present in this compartment in 2009, when she was photographed by a camera-trap set along a trail, no signs of reproduction were documented from 2005 to 2009 in the area known to be occupied by this female from the radio-telemetry and camera-trapping studies indicating a lack of males in this area. The only sign of reproduction that was reported in 2009 is an unconfirmed sighting of a juvenile lynx that was located at the south-western corner of the compartment nearby the border with central Switzerland west (III).

Mortality showed almost the same spatial pattern as reproduction. In pentad 2000–2004 lynx found dead or removed from the population were reported every year only in compartment north-western Swiss Alps. In compartment north-eastern Switzerland they were reported in 2003 in 2004 and in the Valais only in 2004. In the current pentad, with the exception of the Valais from where no mortality was reported, mortality events were additionally reported in compartments central Switzerland west and the Grisons. Although the juvenile lynx found dead in the Grisons originated from the north-eastern Switzerland lynx sub-population (Breitenmoser-Würsten 2009).

The damages to livestock in the current pentad make only one fourth of those reported in the pentad 2000–2004 and were as in the previous pentad mainly located in the north-western Swiss Alps. The possible reasons for this decrease are twofold. First, efficient prevention measures were implemented in the hot spots where damages occurred regularly in the past in the north-western Swiss Alps. Second the roe deer numbers have increased in the north-western Swiss Alps in recent years according to the observations reported by game wardens. Therefore lynx do not need to switch to sheep as they find enough wild ungulates to prey on (Breitenmoser et al. 2010).

Even though the number of lynx presence signs slightly decreased between both pentads, the areas covered by the MCP encompassing all C2 data (27,487 km^2) and the 5-km circular buffer around the C2 data (12,637 km^2) increased by about 36% and 7.6%, respectively. The MCP of the C2 data is about two times larger than the 5-km buffered C2 lynx signs of presence. This discrepancy is due

to the strong fragmentation of the Alps by both artificial and natural barriers. As a consequence the suitable lynx habitat in the Swiss Alps has a patchy distribution (Zimmermann 2004). The MCP approach is not suitable to measure the absolute spatial expansion of the lynx population in this fragmented mountain range as it contains large parts of unoccupied or unsuitable habitat that will never be occupied by lynx. Besides, our results highlighted that it is not suitable to measure the relative changes in the spatial distribution as well as it overestimated the rate of spatial change almost by a factor five. To get a more reliable estimation of the »real« area occupied by the lynx in the fragmented Alpine habitat we buffered the C2 data with a 5-km radius since the last status report (2000–2004; Molinari-Jobin et al. 2006). Although the area resulting from the buffered C2 data collected over a five years period is closer to the »real« spatial distribution of the Alpine lynx population compared to the MCP approach, it does neither take into account imperfect detection into the estimation of the area occupied by lynx nor any dynamic processes such as colonisation and extinction. To palliate these shortcomings we recently started to use a multiple season site occupancy approach to analyse our lynx presence data (Zimmermann et al. in prep.).

Synthesis

The north-western Swiss Alps is still the compartment with the highest number of reported lynx presence signs. As signs of reproduction and mortalities were reported every year we can conclude that the sub-population is functioning well. This compartment is followed by compartments central Switzerland west (III) and north-eastern Switzerland (II) where signs of reproduction and mortalities increased in the last pentad. All three lynx sub-populations acted as source in the current pentad. The translocation to north-eastern Switzerland is still the only significant contribution to the spatial increase of the lynx range in the last 10 years in the whole Alps (Molinari-Jobin et al. 2010). With about 8 independent lynx (KORA unpublished data), this small sub-population is however highly vulnerable. In the context of the Alpine population the north-eastern Switzerland sub-population is very important for the future expansion of the lynx, as it could act as stepping stone to the eastern Alps and could enable to fill the gap towards west (compartment IV). During the current pentad it was documented that at least two individuals already left the compartment: sub-adult male B132 showed the longest dispersal ever reported in the Alps and dispersed over more than 200 km to the Trentino (Haller 2009) and a juvenile lynx died while dispersing in the Grisons (V). However such spontaneous migrations are generally far too rare to allow the establishment of a population and these individuals, if they survive their dispersal, remain isolated for years. However when immigration from different directions is possible – as it is currently the case for compartment central Switzerland West (IV) – the chances that several individuals settle down in a lynx-empty area and start to establish the classical social structure and finally reproduce are improved (Zimmermann et al. 2007). The status of the sub-population in the Valais (VII) is less clear. As almost no signs of reproduction and mortalities were reported over the pentad it acted more as sink than a source. In the remaining compartments there are only a few single individuals that did not yet establish a social structure. An occupancy-based population estimate by Zimmermann et al. (in prep.) based on the ratio of population size estimated by means of photographic capture-recapture analyses and occupancy values of occupied range estimated that about 111 (SE=10) independent lynx lived in the Swiss Alps for the period 2005–2009. This is higher than the 60–90 individuals estimated for the previous period.

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Status of the lynx (*Lynx lynx*) in the German Alps from 2005–2009

Status risa (*Lynx lynx*) v nemških Alpah v obdobju 2005–2009

Sybille Wölfl[a*] and Manfred Wölfl[b*]

[a]Lynx Project Bavaria, Trailling 1a, D – 93462 Lam
[b]Bavarian Conservation Agency, Referat 56 – Landschaftspflege und Wildtiermanagement, Hans-Högn-Straße 12, 95030 Hof/Saale
[*]correspondence: info@luchs.bayern.de

Abstract: We give a short overview of the monitoring results of lynx in the 5-year period 1995–2009. There is no confirmed evidence that there are lynx in the German Alps. Single individuals might have visited the area but signs occur only sporadically. In 2008 Large Carnivore Network has been established to identify and document signs of lynx, wolf and bear. It is the first step to systemize the lynx monitoring. A natural recolonization of the German Alps is not expected in the near future.

Keywords: *Lynx lynx*, status, monitoring, German Alps

Izvleček: Podan je kratek pregled spremljanja stanja risa v petih letih (1995–2009). V tem času ni potrjenih znakov prisotnosti risa v Nemških alpah. Posamezni primerki verjetno obiskujejo območje, vendar se znaki pojavljajo zelo razpršeno. Leta 2008 je bila vzpostavljena »Mreža velikih zveri« za identifikacijo znakov risa, volka in medveda. To je prvi korak k sistemskemu pristopu spremljanja stanja risa. V bližnji prihodnosti ne pričakujemo naravne rekolonizacije risa v Nemških alpah.

Ključne besede: *Lynx lynx*, status, monitoring, nemške Alpe

Introduction

Germany shares an area of around 5.000 km^2 with the Alpine arc which extends to 190.000 km^2 in total. The nearest lynx (*Lynx lynx*) sub-populations to the Germans Alps are found in north-eastern Switzerland (distance 70 km) and in Slovenia (distance 180 km). An evaluation of a possible natural recolonisation of the German Alps concluded a very low probability for establishing a viable population in the next decades (Molinari-Jobin et al. 2010). Even though single dispersers manage to migrate long distances and reach the German Alps it will need a constant flow of lynx dispersing from other sub-populations. A recolonisation of the German Alps will be dependent on expanding sub-populations of Switzerland, Slovenia or Austria or on re-introduction efforts.

Methods

Since 2008 a so called Large Carnivore Network (LCN) is established to identify possible signs of lynx, wolf (*Canis lupus*) and bear (*Ursus arctos*) in the Bavarian Alps. The main focus of the training lies on a thorough documentation to allow verification of signs by experts with long-

term experience. Data which cannot be verified (C3: sightings, all undocumented reports of tracks, kills, calls) are checked for plausibility and then included in or excluded from the data base and classified according to the SCALP categories (Molinari-Jobin et al. in press). To ensure a consistent validation, the data verification in Bavaria is done by two lynx experts who independently evaluate the signs. Thus the data are checked twice and the probability of a misclassification is reduced.

Results and Discussion

The report comprised three 5-year periods of lynx monitoring in the German Alps: 1995–1999, 2000–2004 and 2005–2009 (Kaczensky 1998, Wölfl & Kaczensky 2001, Wölfl 2006, this issue). In the last two periods very few possible lynx signs could be gathered and none of them could be verified or confirmed. Even an assignment to the SCALP category C3 seemed daring because of their very imprecise nature.

During the 2005–2009 period we could only collect very few data as well (n=5, Table 1), all of them were sightings. Four of the chance observations occurred in the western part of the German Alps, Oberallgäu, in the years 2008 and 2009 (Fig. 1). They were accompanied by rumours end of 2009 that a lynx had been shot in that area. One observation stems from the eastern part of the German Alps, Berchtesgadener Land, and occurred in February 2009.

Categories	1995–1999	2000–2004	2005–2009
C1	0	0	0
C2	0	0	0
C3	6	1	5 (all sightings)
Total	6	1	5

Table 1: Number of lynx records collected per period per category (C3: sightings, all undocumented reports of tracks, kills, calls).
Tabela 1: Število zbranih podatkov o znakih prisotnosti risa po obdobjih in po kategorijah (C3: videnja, nedokumentirana opažanja znakov, klicanje).

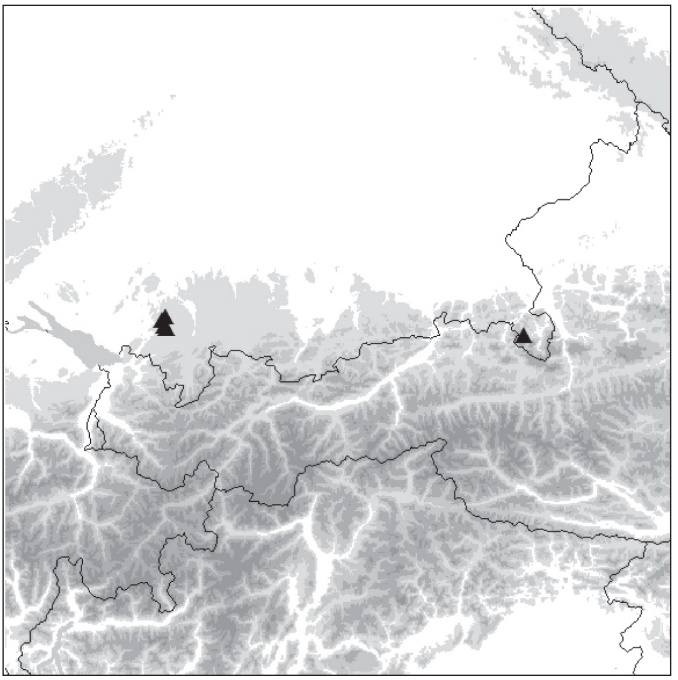

Figure 1: Distribution of lynx signs of presence in the German Alps for the period 2005–2009.
Slika 1: Razporeditev znakov prisotnosti risa v nemških Alpah v obdobju 2005–2009.

It is noticeable that signs in all three periods occurred either in the western part (Allgäu) or in the eastern part (Berchtesgadener Land) of the German Alps. It is therefore possible that there are single dispersers coming from Switzerland or Austria/Slovenia.

With the establishment and training of the LCN in 2008 we have a network of 25 persons in the Bavarian Alps whose awareness is focused on large carnivore signs. This means much better conditions to take notice of signs by checking with local people or even actively looking for signs. Thus the probability of detecting even single lynx should be improved. This assumption is supported by the fact that all lynx signs in the 2005–2009 period had been collected by members of the LCN.

However we have to keep in mind that the presence of observers and a general raise of awareness for large carnivores by the public (due to the presence of a wolf in 2009–2010) could be related to the »increase« of lynx signs. Either this increase is only a function of raised awareness or is substantial, will be confirmed in the future.

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The importance of education of future elementary teachers about modern biotechnology issues

Pomen izobraževanja bodočih učiteljev razrednega pouka o biotehnologiji

Jana Ambrožič-Dolinšek[1*], Andrej Šorgo[2]

[1*]University of Maribor, Faculty of Education, and Faculty of Natural Sciences and Mathematics, Koroška 160, 2000 Maribor, Slovenia
[2]University of Maribor, Faculty of Natural Sciences and Mathematics, Koroška 160, 2000 Maribor, Slovenia
*correspondence: jana.ambrozic@uni-mb.si

Abstract: The tremendous development of science and technology has influenced many aspects of our everyday lives, society and environment. A good example of such technology is biotechnology. However, besides its promise, this technology has also raised several controversial issues to which answers are not easily available. With increasing knowledge and applications on one side and controversy on the other the teaching of science is, anything but easy. Development of competencies for these issues, and questions like why, when, and how to integrate modern biotechnology into science education are becoming prominent in the near future. Nowadays, when we are confronted with issues of varying degrees of complexity and importance, it is necessary that teachers at all levels of education have the basic tools to cope with these issues. This is one of reason why we have attempted to establish what kind of knowledge, values and opinions about genetic engineering and genetically modified organisms (GMOs) are characteristic for the students, future Elementary Teachers, at three Slovene Faculties of Education. We collected answers of 360 questionnaires from pre-service elementary school teachers and analysed their statements from the field of general and classical genetics, modern biotechnology, legislation and the acceptance of different kind of GMOs. Prospective teachers have some knowledge of general and classical genetics and less knowledge about the use of modern biotechnology. They have concerns and fears about different kind of GMOs, mostly negative attitudes towards different kinds of GMOs, or they hold no strong opinions about them. Micro-organisms and plants are generally more acceptable than GM animal. Furthermore, more knowledge does not mean that individual GMOs are more acceptable.

Keywords: genetically modified organisms, GMO, students of elementary education

Abbreviations: GMO – genetically modified organism; GM – genetically modified

Izvleček: Izjemen razvoj znanosti in tehnologije vpliva na številne vidike vsakdanjega življenja posameznika, družbe in okolja. Dober primer tovrstne tehnologije

je biotehnologija. Poleg številnih obetov so s to tehnologijo povezana nekatera sporna vprašanja, na katera ni enostavnih odgovorov. Povečevanje znanj in uporabe na eni in polemik, na drugi strani, je razlog, da je poučevanje biotehnologije vse prej kot lahko. Kako usposobiti bodoče učitelje za obravnavo takih in podobnih tem in zakaj, kdaj in kako vključiti sodobno biotehnologijo v izobraževanje postaja pomembno za bližnjo prihodnost. Zato je nujno, da bi bili učitelji na vseh ravneh izobraževanja usposobljeni za obravnavo takih in podobnih tem. To je bil tudi eden od razlogov, zakaj smo želeli ugotoviti, kakšno je znanje, kakšne so vrednote in mnenja o genskem inženiringu in gensko spremenjenih organizmih (GSO) študentov, bodočih osnovnošolskih učiteljev treh slovenskih pedagoških fakultet (Univerze v Mariboru, Univerze v Ljubljani, Univerze na Primorskem). Zbrali smo odgovore anketnih vprašalnikov 360 bodočih učiteljev razrednega pouka, v katerih so se bodoči osnovnošolski učitelji opredelili do trditev s področja splošne in klasične genetike, moderne biotehnologije, zakonodaje ter sprejemanja različnih GSO. Bodoči učitelji razrednega pouka imajo nekaj znanja o splošni in klasični genetiki in manj znanja o uporabi moderne biotehnologije, velikokrat slabo sprejemajo različne GSO ali nimajo jasno izraženega mnenja o njih, pri čemer so mikroorganizmi in rastline v splošnem bolj sprejemljivi kot GS živali. Več znanja nikakor ne pomeni, da so posamezni GSO bolj sprejemljivi.

Ključne besede: genetsko spremenjeni organizmi, GSO, študenti razrednega pouka

Okrajšave: GSO – genetsko spremenjen organizem; GS – genetsko spremenjen

Introduction

The tremendous development of science and technology has influenced many aspects of our everyday lives, society and environment. A good example of such technology is biotechnology. It is not a recent invention, and humans have used it for centuries. The making of wine, beer, yogurt, cheese and bread, for example, involve ancient biotechnology techniques that have enabled the progress of civilization. Increasing advances in this discipline, such as recombinant DNA technology and the manipulation of genes, as well as the introduction of genes into more or less related organism, the same or different plant and animal species or other organism, to obtain genetically modified organisms (GMOs), have produced many powerful applications and have great potential for future discoveries. However, besides its promise, this technology has also raised several controversial issues (food from GMOs, therapeutic and reproductive cloning, surrogate maternity, potential cloning of people, and the potentially harmful influence of GMOs on the health of people, animals, other organisms and the environment) to which answers are not easily available. The consequence of such issues, called socio-scientific issue (Sadler 2004, Sadler and Zeidler 2005a, Sadler and Zeidler 2005b), is that the transfer of biotechnology discoveries to crop production, industry or medicine is not restricted only by the technological limitations, underdeveloped scientific methods, or modes of scientific reasoning, but also by ethics, morals, faith, the economy, environmental responsibility, risks, politics, etc. (Christoph et al. 2008, Flores and Tobin 2002, Steward and McLean 2005, Yunta et al. 2005). With increasing knowledge and applications on one side and controversy on the other the teaching of science is, anything but easy (Harms 2002). Questions like why, when and how to integrate biotechnology into science education will become prominent in the near future.

The development of opinions and values is a lifelong process originated in early childhood and influenced by school practice; it is not immune to the values, opinions and knowledge of teachers. The formation of values in the case of socio-scientific issues is not at the center of teacher education, and future teachers often construct their

value system about these issues without relevant professional foundations (Ambrožič-Dolinšek and Šorgo 2009). Nowadays, when we are confronted with issues of varying degrees of complexity and importance, it is necessary that teachers at all levels of education should have the basic tools to cope with them (Ambrozic-Dolinsek and Šorgo 2009, 2010).This is one of reason why we have attempted to establish what kind of knowledge, values and opinions about genetic engineering and genetically modified organisms (GMOs) are characteristic of students, future elementary teachers at three Slovene Faculties of Education: University of Maribor (PeFMb), University of Ljubljana (PeFLj) and University of Primorska (PeFKp). Our results could potentially be included in the undergraduate curriculum for the education of future and current elementary teachers.

Material and methods

We collected 360 questionnaires from students, future elementary teachers at three Slovene Faculties of Education (University of Maribor (PeFMb), University of Ljubljana (PeFLj) and University of Primorska (PeFKp)) in the academic year 2007/2008.

To find out student teachers' knowledge and opinion about GMOs, a questionnaire was assembled. The questionnaire was divided into two parts: (1) knowledge, and (2) acceptance about GMO and was completed anonymously. Knowledge concerning genetics, biotechnology and GMO was evaluated through a questionnaire consisting of 30 true–false statements (Table 1). Teachers had to choose among three options: yes; do not know; no.The correct answer on 17 statements was 'yes' and on 13 statements 'no', a device which prevented guessing. The statements could be assigned to general and classical genetics, modern biotechnology and legislation. The reliability of the questionnaire, expressed as Cronbach's alpha, was 0.827, which can be recognized as good. In Table 1 frequencies and percentages of correct, incorrect, and do not know answers are reported.

Furthermore we tried to establish the degree of acceptance of different kinds of GMO uses in possible real life situations, so we provided statements about various GMOs – microorganisms, plants and animals (Table 2). Acceptance of GMOs was evaluated with a closed questionnaire, where teachers were asked to choose among 17-items consisting of existing or potentially-existent GMOs and in such way to express their opinion about these. We provided three answers: 1- acceptable; 2 – don't know, do not have an opinion; 3 – not acceptable. The reliability of the questionnaire, expressed as Cronbach's alpha, was 0.869, which can be recognized as good.

Analysis of the results followed three tracks and the statistical package SPSS® 18.0 was used for data analysis. Chi-square (χ^2) statistics were used to identify differences in frequencies of answers from two general fields: first from the statements from general genetics and the statements from classic and modern biotechnology and legislation and the second from statements about acceptance of different kind of GMOs. To correlate their answers, the Pearson correlation coefficient was used. Symbols used in the figures are: ns denote statistically insignificant difference.

Results and discussion

Future elementary school teachers from three Slovenian universities (University of Maribor, University of Ljubljana, and University of Primorska) do have some basic knowledge of genetics (Table 1). They possess at least some knowledge about classical genetics and know something about genes, their structure, replication, expression and mutations. The majority of them correctly determined 9 among 14 (64.3%) statements, incorrectly determined 2 among 14 (14.3%) statements and do not know 3 among 14 (21.4%) statements. However, we should not be satisfied with observed knowledge. For example, some of them believe that a cat can fertilize a female rabbit, and they do not know that the broad use of vegetative propagation in plants is a kind of cloning.

The picture changed when they had to choose the correct statements in the areas of modern biotechnology and legislation. We observed deficiencies in their knowledge about current applications of modern biotechnology, such as transmission of genes between organisms,

Table 1: Knowledge of future elementary teachers from three Slovene Faculties of Education about genetically modified organisms. The highest frequencies of answers for individual statement are in bold.
Tabela 1: Znanje bodočih učiteljev razrednega pouka s treh Slovenskih pedagoških fakultet. Najvišje frekvence so označene s pisavo krepko.

	Statement	Correct answer	YES		NO		Do not know/ empty	
			N	%	N	%	N	%
	Knowledge about classical genetics							
1	Bacteria have the ability to mutually exchange genes.	Yes	52	15.2	46	13.5	243	71.3
3	Deoxyribonucleic acid (DNA) occurs only in genetically modified organisms.	No	13	3.8	**215**	**62.9**	114	33.3
4	Bacteria genes from yogurt that can be consumed can be incorporated into cells in the human organism.	No	45	13.2	119	34.8	**178**	**52.0**
5	Genes are sequences (of nucleotides) on chromosomes.	Yes	**183**	**53.5**	42	12.3	117	34.2
6	Genes are not normally transmitted from species to species in nature.	Yes	87	25.4	**166**	**48.5**	88	25.8
10	A cat can fertilize a female rabbit; the resulting young rabbits have shorter ears.	No	10	2.9	**227**	**66.4**	105	30.7
11	Mutations are the result of cloning.	No	105	30.7	**58**	**46.2**	79	23.1
12	Mutations are always inherited.	No	60	17.5	**185**	**54.1**	97	28.4
13	Deoxyribonucleic acid (DNA) is a source of information for the synthesis of proteins.	Yes	**190**	**55.4**	15	4.5	132	39.2
18	Propagation of plants by cuttings is cloning.	Yes	56	16.5	**220**	64.7	64	18.8
19	Recessive genes are never expressed.	No	18	5.3	85	25.1	**236**	**69.6**
22	The sex of the child depends on male sex cells.	Yes	**223**	**65.2**	79	23.1	40	11.7
25	All mutations are harmful.	No	36	10.6	**225**	**66.0**	80	23.5
26	Bread rising is a biotechnological process.	Yes	**102**	**30.3**	87	25.8	148	43.9
	Knowledge about current applications of modern biotechnology							
2	The vaccine against hepatitis B used to vaccinate all school children was produced with genetically modified yeast.	Yes	33	9.6	36	10.5	**273**	**79.8**
7	GM crops are cultivated in Slovenia.	No	**200**	**58.7**	17	5.0	124	36.4
8	Insulin for treating human diabetes is produced from GM (genetically modified) pig and cow pancreata.	No	25	7.3	39	11.4	**278**	**81.3**
9	Products from GMO (genetically modified organisms) must be labeled as containing GM components.	Yes	**239**	**70.3**	18	5.3	83	24.4
14	Before application of GM (genetically modified) plants, it is obligatory to perform a risk assessment about possible harmful influences of GM plants on the health of people, animals (other organisms) and the environment.	Yes	**229**	**67.0**	11	3.2	102	29.8
15	Reproductive cloning from cells harvested from an adult produces an embryo from which develops a child genetically identical to this adult.	No	**183**	**53.5**	22	6.4	137	40.1

	Statement	Correct answer	YES		NO		Do not know/ empty	
			N	%	N	%	N	%
17	Therapeutic cloning from stem cells harvested from an adult produces several types of cells used for treating diseases or harmful tissues of the same person.	Yes	98	28.7	20	5.8	**224**	**65.5**
20	Ribonucleic acid (RNA) is a genetically modified form of deoxyribonucleic acid (DNA).	No	29	8.5	147	43.0	166	48.5
21	Slovenia has passed a law dealing with GMOs.	Yes	51	8.5	31	43.0	**258**	**48.5**
23	Biogas methane from biogas reactors is produced by bacteria.	Yes	39	11.5	20	5.9	280	82.6
24	In Slovenia only GM corn is produced and marked as MON 810.	No	17	5.0	41	12.0	**283**	**83.0**
27	The cloning of genes and the cloning of organisms require the same methods of work.	No	41	12.0	67	19.6	**234**	**68.4**
28	Stem cells occur in adult humans.	Yes	**156**	**45.7**	19	5.6	166	48.7
29	Cloning of human embryos is already possible.	Yes	**192**	**56.3**	52	15.2	97	28.4
30	The transfer of animal genes to plants is possible.	Yes	44	12.9	87	25.4	**211**	**61.7**

production of medicines with GMOs, cloning of organisms and about GMO legislation, and the cultivating of GM crops in Slovenia. The majority of them correctly determined 5 among 16 (31.0%) statements, incorrectly determined 2 among 16 (12.0%) statements and do not know 9 among 16 (56.2%) statements.

Comparison of »do not know« with »yes« and »no« statements showed statistically significant higher number of »do not know« statements ($\chi^2 = 188.283$, $h = 4$, $p > 0.001$) about current applications of modern biotechnology, then about classical genetics. The high percentages of »do not know« answers indicate that they are aware of their insufficient knowledge about modern biotechnology. This could mean that future elementary teachers need additional more biotechnology topics in their education.

School practice is not completely impervious to the knowledge, values, opinions and attitudes of teachers. In other words, teacher's values, opinions and attitudes can play a certain role in the acceptance of biotechnology issues by school pupils by the whole vertical of compulsory education. Attitudes toward genetic modified organisms among students, future elementary teachers at three Slovene Faculties of Education were already evaluated and analysis of their answers reveals uncertainty, distrust and rejection (Ambrožič-Dolinšek and Šorgo 2009). The same is true for acceptance of different kind of GMOs. Among 17 different kinds of GMOs, only 5 are acceptable to more than 50% of students; students either find others not acceptable or have no opinion (Table 2). This low level of acceptance again indicates that in most cases, the attitudes of future elementary school teachers from three Slovenian universities toward GMOs are not positive or they hold no strong opinions about them.

In dealing with acceptance, we were able to recognize two patterns. The first one is that GM microorganisms and plants are generally more acceptable than GM animals, which are actually unacceptable. Our results confirm that acceptance of one type of GMO does not mean that some other GMO will also be acceptable (Steward and McLean 2005). The second pattern is that GMOs not used for food consumption are generally more acceptable if they or their parts cannot be used directly or indirectly for consumption and if they produce something recognized as useful for purposes such as medicine, bio-fuel, or organic substances, and have the capacity to clean something, or to improve resistance to stress conditions. A drop in the level of acceptance in pairs was observed, where plants tolerant to stress are acceptable to more than half the teachers, while plants manipulated to be tolerant to pests in food production are ac-

ceptable to only one-third of respondents. Among plants, the lowest scores were given to ornamental plants, a result which can be connected with the level of perceived utility and benefit. Genetically manipulated animals, always in the lower ranks of acceptability, are especially unacceptable if they have been manipulated for food consumption. The lowest scores in acceptability were given to genetically modified viruses. We can speculate that the answers somehow correlate with knowledge of and attitudes towards viruses as the cause of disease, which is never recognized as useful. In the uncertainty group (do not know; do not have an opinion), there occurred only microorganisms and viruses, which crossed the fifty percentages border. Students cannot decide whether or not manipulated viruses and microorganisms modified for production of substances for the food industry and synthesis of organic substances are acceptable. An interesting issue is their relation to health. It seems that, in the case of health, GMO plants and microorganisms could become more acceptable. When human health is at issue, the acceptance level of GMOs appears higher, as has also been shown by other studies (Cavanagh et al. 2005).

The correlation among knowledge and acceptance level was calculated. There was no correlation between knowledge and acceptance ($r = 0{,}052^{ns}$). It seems that GMOs acceptance is not connected with more knowledge or more knowledge about genetics does not automatically mean that GMOs would be more accepted.

Biotechnology is in broader sense the use of living organisms to solve problems and make useful products and applications (Thieman and Palladino 2009) and intended to improve the quality of human life. Currently we are witness of public resistance and skepticism to science, especially to modern biotechnology. Some assign it to the low levels of knowledge of science or »scientifically illiterate« public (Allum et al. 2008) and the importance of introduction of biotechnology in the education at the whole vertical of undergraduate curriculum. Education should starts with introduction of the science behind simply everyday biotechnology practices as making of food stuff like cheese and bread and continues with other more sophisticated agronomy, food and drink producing practices later continuing with some modern biotechnology practices.

Our study shows that there is no correlation between knowledge and acceptance of GMOs, and the former studies (Šorgo and Ambrožič 2009, 2010) that there it is strong correlation between acceptance and attitudes against GMOs, meaning that attitudes and not knowledge shaped the acceptance. So simple introduction of biotechnology, and science behind, by addition of new facts or teacher-provided explanations about ancient and current biotechnological processes does not influence the acceptance.

Public resistance and skepticism to science mean that modern biotechnology is not recognized only as something beneficial. Especially popular media sometimes present it as a threat, or controversial issue, causing concerns in society (Šorgo et al. 2011). Schools and teachers, as a part of society, must be prepared also for dealing with such socio-scientific issues and should be trained to developed competences based on active work of pupils such as critical thinking or scientific reasoning of pros and contra.

Emotions are especially important part of elementary education (Čagran et al. 2008) and could be important factor in shaping attitudes toward different GMOs ant their acceptability (Šorgo et al. 2011). Emotions related to GMOs are usually negative and hidden in concerns, risk, uncertainty, worry, anger and fear (Šorgo et al. 2011), and the same pattern was observed in emotions expressed by our future teachers. Negative emotions of future teacher against modern biotechnology, no matter of their origins, would not supported and lead to higher acceptance of this technology. This also supported the need for early introducing of biotechnology in education, development of positive experiences with biotechnology and also the importance of education of competent future and current elementary teachers.

Conclusions

The students included in our study have concerns and fears about different kind of GMOs and mostly negative attitudes towards different kinds of GMOs, or they hold no strong opinions about them. Only a few of GMOs are accepted by more than half the students. We also observed some knowledge (often severely flawed) about

classical genetics and little or no knowledge about current applications of modern biotechnology and the last is not differing from other publics (Allum et al. 2008). The early positive experiences with biotechnology are recommended. Schools and teachers, as a part of society, must be prepared also for dealing with socio-scientific issues.

Povzetek

Izjemen razvoj znanosti in tehnologije vpliva na številne vidike vsakdanjega življenja posameznika, družbe in okolja. Dober primer tovrstne tehnologije je biotehnologija. Poleg številnih obetov so s to tehnologijo povezana nekatera sporna vprašanja, na katera ni enostavnih odgovorov. Povečevanje znanja in uporabe na eni ter polemik, na drugi, dela poučevanje biotehnologije vse prej kot lahko. Kako usposobiti bodoče učitelje za obravnavo takih tem in zakaj, kako in kdaj vključiti sodobno biotehnologijo v izobraževanje bo postalo pomembno v bližnji prihodnosti. Pomembno je, da bi bili učitelji vseh ravni izobraževanja usposobljeni za obravnavo takih in podobnih tem. To je bil tudi eden od razlogov, zakaj smo želeli ugotoviti, kakšno je znanje, kakšne so vrednote in mnenja o genskem inženiringu in gensko spremenjenih organizmih (GSO) študentov, bodočih osnovnošolskih učiteljev treh slovenskih pedagoških fakultet (Univerze v Mariboru, Univerze v Ljubljani, Univerze na Primorskem). Zbrali smo odgovore anketnih vprašalnikov 360 bodočih učiteljev razrednega pouka, v katerih so se bodoči osnovnošolski učitelji opredelili do trditev s področja splošne in klasične genetike, moderne biotehnologije, zakonodaje ter sprejemanja različnih vrst GSO. Bodoči učitelji razrednega pouka imajo kar nekaj znanja o splošni in klasični genetiki, čeprav z doseženim ne moremo biti povsem zadovoljni. Zelo šibko je njihovo znanje o uporabi moderne biotehnologije in z njo povezano zakonodajo. Bodoči učitelji zelo slabo sprejemajo različne GSO ali nimajo svojega mnenja o njih. GS mikroorganizmi in rastline so v splošnem bolj sprejemljivi kot GS živali. Pri tem so še posebej nesprejemljive GS živali za hrano. Ko gre za zdravje so GS mikroorganizmi in rastline bolj sprejemljive. Med znanjem in sprejemanjem GSO ni korelacije, kar pomeni, da več znanja nikakor ne pomeni, da bodo posamezni GSO bolj sprejemljivi.

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INSTRUCTIONS FOR AUTHORS

1. Types of Articles

SCIENTIFIC ARTICLES are comprehensive descriptions of original research and include a theoretical survey of the topic, a detailed presentation of results with discussion and conclusion, and a bibliography according to the IMRAD outline (Introduction, Methods, Results, and Discussion). In this category ABS also publishes methodological articles, in so far as they present an original method, which was not previously published elsewhere, or they present a new and original usage of an established method. The originality is judged by the editorial board if necessary after a consultation with the referees. The recommended length of an article including tables, graphs, and illustrations is up to fifteen (15) pages; lines must be double-spaced. Scientific articles shall be subject to peer review by two experts in the field.

REVIEW ARTICLES will be published in the journal after consultation between the editorial board and the author. Review articles may be longer than fifteen (15) pages.

BRIEF NOTES are original articles from various biological fields (systematics, biochemistry, genetics, physiology, microbiology, ecology, etc.) that do not include a detailed theoretical discussion. Their aim is to acquaint readers with preliminary or partial results of research. They should not be longer than five (5) pages. Brief note articles shall be subject to peer review by one expert in the field.

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2. Originality of Articles

Manuscripts submitted for publication in *Acta Biologica Slovenica* should not contain previously published material and should not be under consideration for publication elsewhere.

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Articles and notes should be submitted in English, or as an exception in Slovene if the topic is very local. As a rule, congress and association news will appear in Slovene.

4. Titles of Articles

Title must be short, informative, and understandable. It must be written in English and in Slovene language. The title should be followed by the name and full address of the authors (and if possible, fax number and/or e-mail address). The affiliation and address of each author should be clearly marked as well as who is the corresponding author.

5. Abstract

The abstract must give concise information about the objective, the methods used, the results obtained, and the conclusions. The suitable length for scientific articles is up to 250 words, and for brief note articles, 100 words. Article must have an abstract in both English and Slovene.

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There should be no more than ten (10) keywords; they must reflect the field of research covered in the article. Authors must add keywords in English to articles written in Slovene.

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The introduction must refer only to topics presented in the article or brief note.

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Articles should not contain more than ten (10) illustrations (graphs, dendrograms, pictures, photos etc.) and tables, and their positions in the article should be clearly indicated. All illustrative material should be provided in electronic form. Tables should be submitted on separate pages (only horizontal lines should be used in tables). Titles of tables and illustrations and their legends should be in both Slovene and English. Tables and illustrations should be cited shortly in the text (Tab. 1 or Tabs. 1-2, Fig. 1 or Figs. 1-2; Tab. 1 and Sl. 1). A full name is used in the legend title (e.g. Figure 1, Table 2 etc.), written bold, followed by a short title of the figure or table, also in bold. Subpanels of a figure have to be unambiguously indicated with capital letters (A, B, …). Explanations associated with subpanels are given alphabetically, each starting with bold capital letter (**A**), a hyphen and followed by the text.

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Articles shall end with a summary of the main findings which may be written in point form.

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Articles written in Slovene must contain a more extensive English summary. The reverse also applies.

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References shall be cited in the text. If a reference work by one author is cited, we write Allan (1995) or (Allan 1995); if a work by two authors is cited, (Trinajstić and Franjić 1994); if a work by three or more authors is cited, (Pullin et al. 1995); and if the reference appears in several works, (Honsig-Erlenburg et al. 1992, Ward 1994a, Allan 1995, Pullin et al. 1995). If several works by the same author

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Books, chapters from books, reports, and congress anthologies use the following forms:

Allan, J.D., 1995. Stream Ecology. Structure and Function of Running Waters, 1st ed. Chapman & Hall, London, 388 pp.

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A manuscript element checklist (For a manuscript in Slovene language the same checklist is appropriately applied with a mirroring sequence of Slovene and English parts):
English title – (Times New Roman 14, bold)
Slovene title – (Times New Roman 14, bold)

Names of authors with clearly indicated addresses, affiliations and the name of the corresponding author – (Times New Roman 12)
Author(s) address(es) / institutional addresses – (Times New Roman 12)
Fax and/or e-mail of the corresponding author – (Times New Roman 12)
Keywords in English – (Times New Roman 12)
Keywords in Slovene – (Times New Roman 12)
Running title – (Times New Roman 12)
Abstract in English (Times New Roman 12, title – Times New Roman 14 bold)

Abstract in Slovene – (Times New Roman 12, title – Times New Roman 14 bold)
Introduction – (Times New Roman 12, title – Times New Roman 14 bold)
Material and methods – (Times New Roman 12, title – Times New Roman 14 bold)
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Figure legends; each in English and in Slovene – (Times New Roman 12, title – Times New Roman 14 bold, figure designation and figure title – Times New Roman 12 bold)
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Acknowledgements – (Times New Roman 12, title – Times New Roman 14 bold)
Literature – (Times New Roman 12, title – Times New Roman 14 bold)
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