

## Evidence of hybridization between *Galatella villosa* and *G. linoisyris*, and a taxonomic reappraisal of the hybrid *G. ×subvillosa*

Důkaz hybridizace mezi *Galatella villosa* a *G. linoisyris*, a taxonomické přehodnocení křížence *G. ×subvillosa*

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At the westernmost distribution of the steppe herbaceous plant, *Galatella villosa*, in Hungary, Serbia and Ukraine, we recently observed intermediate specimens between this species and its close relative, *G. linoisyris*. We were able to demonstrate the hybrid origin of these individuals by sequencing the biparentally inherited nuclear ribosomal internal transcribed spacer (nrITS) region and checking additive polymorphism in the hybrids. In addition, examination of the maternally inherited plastid regions (*trnH-psbA* and *trnL-trnF* intergenic spacers) revealed that *G. villosa* is likely to be the maternal parent in the Hungarian and Ukrainian populations and *G. linoisyris* in the Serbian population. The intermediate forms produced only sterile seeds. The alleged hybrid between the above two species has already been described as *G. ×subvillosa* based on a very brief diagnosis. Still, the analysis of the morphological characters using linear discriminant analyses clearly separated the holotype of *G. ×subvillosa* from individuals of *G. linoisyris* × *G. villosa*. The latter appeared to be morphologically intermediate between populations of *G. villosa* and *G. linoisyris*. Contrary to the originally stated hybrid origin of the type plants of *G. ×subvillosa*, morphological evidence indicates the involvement of *G. divaricata* not *G. linoisyris*. The hybrid *G. linoisyris* × *G. villosa* is thus described here, as a new nothospecies *G. ×feketegaborii*. This study highlights the power of easily available molecular phylogenetic tools for demonstrating the hybrid origin of plants and illustrates how additive polymorphism can be distinguished from other types of intraindividual polymorphism in nuclear DNA sequences.

Keywords: *Astereae*, additive polymorphic site (APS), bidirectional hybridization, *Compositae*, hybrid sterility, multivariate morphometrics, nothospecies

### Introduction

Cross-fertilization is a general and widely recorded phenomenon in the vascular plant family *Asteraceae* (*Compositae*) with several examples of interspecific (e.g. Guo et al.

2005, Mráz et al. 2005, Roché & Susanna 2010) and even intergeneric hybridizations (e.g. Li 2006, Fehrer et al. 2007, Saito et al. 2007, Freire 2012). Past hybridization events led to reticulate evolution in the phylogenetic history of this family (Jones & Young 1983, Guo et al. 2005, Fehrer et al. 2007), whereas more recent processes led to the formation of primary hybrids (Mráz et al. 2005, Li 2006, Saito et al. 2007, Roché & Susanna 2010). Such plants are usually morphologically intermediate, but intermediate morphology is not necessarily indicative of hybrid origin (Řepka et al. 2014).

The Eurasian genus *Galatella* Cass. (Nesom & Robinson 2007) of the family *Asteraceae* is sometimes included in the genus *Aster* L. (in a broad sense). Nevertheless, recent phylogenetic studies (Li et al. 2012, Jafari et al. 2015, Korolyuk et al. 2015) clearly demonstrate the polyphyly of *Aster* s.l., support the splitting-off of *Galatella* and reject further splitting of the “*Galatella* group” into separate genera (i.e. *Linosyris* Cass. and *Crinitina* Soják). Therefore, in this study, we follow the broad circumscription of *Galatella* as a genus distinct from *Aster*.

Several nothospecies of the otherwise Eurasian *Galatella* are described from Russia, including *Galatella* *xsubtatarica*, *G. xsublinosyris* and *G. xsubvillosa* by Tzvelev (1994), and *G. xtzvelevii* by Vasjukov et Saksonov (2015). Tzvelev (1959, 1994) also suggests the hybrid origin of *G. crinitoides* Novopokrovsky. Our general knowledge of Tzvelev’s nothotaxa is, however, rather imperfect. Although Tzvelev’s original diagnoses are sufficient for the purpose of valid publication according to Turland et al. (2018), they are minimalistic and superficial, being confined to a really short (i.e. one line) morphological statement, distinguishing a hybrid from one of its putative parents. Recognition of the three hybrid species is based on single herbarium sheets collected by D. E. Janischewsky in 1912 and 1913 from the vicinity of the town Saratov in Russia, which are kept at the Komarov Botanical Institute, St. Petersburg (LE) (Tzvelev 1994). These specimens were designated by Tzvelev as nomenclatural types of the nothospecies.

*Galatella xsubvillosa* Tzvelev is described as a spontaneous hybrid between *G. villosa* (L.) Rchb. f. and *G. linosyris* (L.) Rchb. f. The former species is a typical steppe plant distributed from south-western Siberia to eastern Europe (Tzvelev 1959, Meusel & Jäger 1992), whereas the latter is recorded in temperate Europe from the Caucasus Mountains and the Volga River in the east to the Atlantic coast in the west (Tzvelev 1959, Meusel & Jäger 1992). Distributions of the two species extensively overlap where the forest-steppe and steppe zones meet. Although each species can be common locally in its subzone in the steppe region (i.e. *G. villosa* is rather common on the steppes of Ukraine and Russia, *G. linosyris* is widespread in the forest-steppe zone from Hungary to the middle course of the Volga River), there are isolated occurrences of both species in the other’s zones (Fig. 1). The western outposts of *G. villosa* are isolated and as such are valued from a conservation point of view (Stevanović 1999, Király 2009).

At the two westernmost localities of *G. villosa* in Europe, the village of Tarcal in Hungary and Krušće in Serbia, we found specimens of *Galatella* morphologically intermediate between *G. villosa* and *G. linosyris*. Similar individuals were also found at Yelanetskyi Step (Mykolaiv Oblast, Ukraine). In this study, we aim at (i) testing the hybrid nature of the apparently intermediate specimens using molecular phylogenetic markers, and (ii) unravelling the morphological relationships between these taxa.

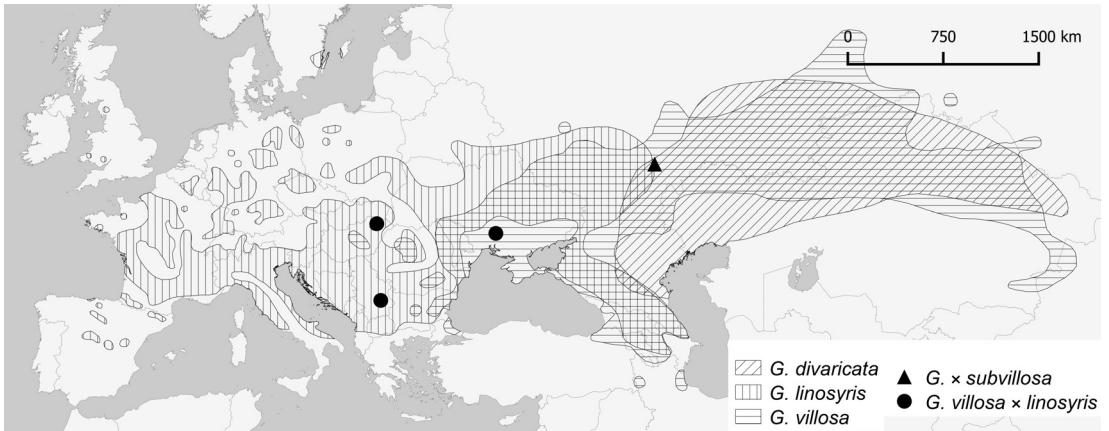


Fig. 1. – Map showing the distributions of the three species of *Galatella* included in the present study (Meusel & Jäger 1992, redrawn). Symbols highlight the populations sampled.

## Materials and methods

### *Molecular test of hybridity*

For molecular genetic analyses, tissue samples of *G. linosyris*, *G. villosa* and the putative hybrid were collected in the vicinity of the village Tarcal (north-eastern Hungary, Borsod-Abaúj-Zemplén County, ~200 m a.s.l., 48.111°N, 21.367°E), from the village Krušce (southern Serbia, Nišava District, ~290 m a.s.l., 43.332°N, 21.742°E) and on the Yelanetskyi Step (southern Ukraine, Mykolaiv Oblast, ~70 m a.s.l., 47.566°N, 32.023°E). One individual of the parents and two of the putative hybrids per population were sampled. The tissue samples were dried and stored in silica-gel until processed.

Whole genomic DNA was extracted by using a modified version of the cetyltrimethylammonium bromide extraction protocol of Sramkó et al. (2014). The hybrid origin of the plants was confirmed by using a biparentally inherited molecular genetic region, the nuclear ribosomal internal transcribed spacer (nrITS), following the results of Fuertes Aguilar et al. (1999), who demonstrate the additivity of polymorphic sites in this marker in  $F_1$  hybrids resulting from the co-amplification of parental copies in their hybrids. To trace the ovule donor of the supposed hybrid plants, we also sequenced two plastid-encoded regions, the *trnH-psbA* intergenic spacer (IGS) and the *trnL-trnF* IGS.

The amplification of the nrITS region in a polymerase chain reaction (PCR) followed the procedure described by Sramkó et al. (2014). We used the primers ITS1A (Sramkó et al. 2014) and ITS4 (White et al. 1990) in a standard PCR-mixture containing bovine serum albumin (BSA) and DreamTaq Green polymerase (Thermo Scientific, USA). Sequencing of the PCR-products was done by Macrogen Inc., Korea, using the original primers as sequencing primers. As for the plastid regions, we used the primers used by Sang et al. (1997) to amplify *psbA-trnH*, whereas we used the ‘c’ and ‘f’ primers of Taberlet et al. (1991) for *trnL-trnF*. PCR-amplification followed the procedure described in Sramkó et al. (2014). We used the same touchdown PCR regime to amplify the plastid regions using a standard PCR-mixture with BSA and DreamTaq Green polymerase

(Thermo Scientific, USA). Sequencing again was done by Macrogen Inc. (Korea) using both original primers in both regions.

Sequence reads in both directions (i.e. forward and reverse) were carefully examined for peak additivity in the nrITS region using ChromasLITE v.2.6.2 (Technelysium Pty. Ltd., Australia). Such sites were coded by IUPAC ambiguity symbols for the coexistence of different nucleotides in the same nucleotide (nt) position. An additive peak was only accepted as such if the secondary peak was (i) present in both forward and reverse reads, and (ii) its height exceeded 25% of the other peak (see also Fuertes Aguilar et al. 1999). It is important, however, to make a distinction between site additivity due to hybridity and the molecular evolutionary dynamics of the region (e.g. the presence of paralogous copies as a result of molecular evolutionary processes; Whittall et al. 2000, Fuertes Aguilar & Nieto Feliner 2003). In this respect, we adopt the approach of Fuertes Aguilar & Nieto Feliner (2003), who defined additive polymorphism of hybrids as “additive polymorphic site (APS): when the two bases involved in a polymorphic site were also found separately in other accessions of the data set”, thus, excluding additivity due to paralogy, which is widespread in nrITS (Bailey et al. 2003, Nieto Feliner & Rosselló 2007). For plastid sequences, forward and reverse reads were used to make a ‘contig’ sequence of the sample. All DNA-sequences generated for this study are deposited in GenBank (accession numbers: MT682313–MT682336 & MT703636–MT703647). Finally, the two plastid regions were combined into a concatenated plastid sequence. Given the slight divergence between the taxa included in the molecular analyses, we were able to align the sequences by eye in BioEdit v7.1.3 (Hall 1999).

### *Morphometric approach*

Nine morphological characters (Table 1) of the putative hybrid were measured in the field, in the Hungarian and Serbian populations (the Ukrainian population was excluded because of the poor condition of the individuals). The same traits of the parents were measured on high-resolution scanned images (by using ImageJ v.1.51; Schneider et al. 2012) of herbarium sheets from Russian herbarium (MW) and from central Europe (BP, DE). We included the holotype specimen of *G. xsubvillosa* (LE01010256) (Fig. 2). We measured only flowering individuals with complete foliage. More than one shoot from a herbarium sheet was included only if separate shoots were mounted on the sheet (possibly representing independent individuals). Barcodes of all specimens used are listed in Appendix 1.

To decide whether the individuals considered to be hybrids are sterile or fertile (able to reproduce even by backcrossing with the putative ascendants), achenes collected from the Hungarian populations were examined by eye. The pollen grains of the taxa involved were checked on herbarium specimens using a Carl Zeiss (Jena) microscope at 1000× magnification using a HI 100×/1.25 objective with oil immersion. Digital photographs were taken using a Canon 2000D camera.

Linear discriminant analysis (LDA) was used to reveal the morphological differences between the taxa studied. Within the LDA we used established taxonomic names as predefined groups, except for *G. villosa* and *G. linosyris*, where we defined two geographically distinct groups (EE = eastern Europe, CE = central Europe). For the sake of simplicity, we included the Serbian population in group CE although we accept it should geographically be classified as south-eastern Europe (Brummitt 2001). LDA was used to visualise the





Fig. 2. – Type specimen of *Galatella xsubvillosa* Tzvelev (LE01010256)

Table 1. – Morphological characters (mean±S.D.) of the *Galatella* taxa studied. Geographic origin of groups of samples is indicated as CE for central Europe and EE for eastern Europe. Herbarium samples used for obtaining these measurements are listed in Appendix 1.

Taxon	n	Height of the flowering stem (mm)	Number of leaves	Width of lower leaves (mm)	Length of lower leaves (mm)	Width of middle leaves (mm)	Length of middle leaves (mm)	Width of upper leaves (mm)	Length of upper leaves (mm)	Number of capitula
<i>G. villosa</i> EE	10	201.0±39.4	17.4±4.6	4.4±1.1	23.7±4.2	4.2±0.8	19.5±2.7	2.6±0.4	13.8±1.4	13.1±3.6
<i>G. villosa</i> CE	10	270.7±50.9	16.8±5.3	4.4±1.6	22.6±6.7	4.0±1.3	21.4±5.6	2.8±1.4	16.7±4.8	7.0±1.6
<i>G. linosyris</i> EE	10	421.2±102.2	70.0±27.0	1.4±0.6	41.8±5.2	0.8±0.2	34.4±5.8	0.5±0.2	19.3±4.4	7.8±4.7
<i>G. linosyris</i> CE	10	395.7±80.5	85.5±13.0	1.4±0.2	36.4±6.5	0.9±0.3	29.0±6.8	0.6±0.1	16.3±2.4	7.1±2.5
<i>G. villosa</i> × <i>G. linosyris</i>	34	401.6±43.5	37.7±9.7	3.8±0.7	42.2±5.5	2.3±0.5	31.9±3.7	1.7±0.3	23.6±7.3	9.6±7.8
<i>G. xsubvillosa</i>	6	341.5±16.5	15.0±6.2	2.2±0.2	28.1±2.2	2.1±0.1	29.4±3.3	1.9±0.4	28.0±3.4	25.5±6.9

morphological differentiation across the taxa studied. In order to quantify the appropriate classification of the LDA, we employed a leave-one-out cross-validation. During this validation, one known specimen is removed from the LDA at a time, and assigned using the discriminant function calculated based on all the cases except the removed specimen. The percentage of correct assignments indicate the reliability of the discriminant function.

In order to statistically test the morphological differences across the taxa studied we used permutational multivariate analysis of variance (PERMANOVA, Anderson 2001), as some measured characters had non-Gaussian distributions. This procedure is a semi-parametric method of multivariate analysis of variance (i.e. MANOVA), being based on pairwise dissimilarities between the groups studied and is largely unaffected by heterogeneity of variances, unbalanced sampling design or non-Gaussian character distributions. PERMANOVA was performed based on pairwise Gower distances, as the used characters included both continuous and discrete traits, and the final estimates were based on 10,000 permutations. All P-values were corrected for multiple comparisons using the Bonferroni correction procedure. LDA was performed as implemented in R package MASS (Venables & Ripley 2002) in R version 3.5.2 (R Core Team 2018), while PERMANOVA was carried out in PAST version 3.26 (Hammer et al. 2001).

## Results

### *Molecular genetic evidence*

Sequences of nrITS were 630 base pairs (bp) long in *G. villosa*, but one base shorter in the other supposed parental species, *G. linosyris* (629 bp): indel of a thymine (T) nucleotide at position 521 was present in all *G. villosa* samples (Table 2). There were 33 variable sites in the nrITS region (16 in ITS1, none in 5.8S, and 17 in ITS2) in the two supposed parental species. We detected additive polymorphism in both putative parental sequences indicative of the presence of intraindividual sequence variants (including paralogous and incompletely homogenized nrITS copies) (Table 2).

Samples of the supposed hybrid displayed peculiar additivity of nrITS sequences of *G. villosa* and *G. linosyris*. First, a clearly additive pattern of the parental nrITS copies was identified using APSS; at 13 variable sites that separate the parental species, all putative

Table 2. – Variable sites in the nrITS sequences of *Galatella linosyris* Gl, *G. villosa* Gv and their putative hybrids Gv × Gl. Additive polymorphic sites, which demonstrate hybridity as defined in the text, are highlighted with asterisks. Sample provenance is indicated by ISO country codes.

Sample	nucleotide position of nrITS sequence																																								
	ITS1											ITS2																													
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*																		
	1	6	8	9	9	0	2	2	3	3	9	0	2	2	3	3	3	3	4	4	4	5	3	3	4	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5
	8	8	1	7	9	9	3	4	4	7	3	0	2	4	4	5	5	6	1	1	4	7	8	6	9	1	4	9	7	3	4	6	0	2	2	2	2	2	2	2	
Gl HU	G	M	A	G	A	T	C	A	T	Y	T	R	T	T	T	T	T	C	G	C	T	R	A	C	T	-	A	C	R	G	C	C	A	C	A	C	A	C	A	C	
Gl RS	G	M	A	G	A	T	C	A	T	C	T	R	T	T	T	T	T	C	G	C	T	G	A	C	T	-	A	C	R	G	C	C	A	C	A	C	A	C	A	C	
Gl UA	G	M	A	G	A	T	C	A	T	C	T	G	T	T	T	Y	Y	Y	G	C	T	R	A	C	T	-	R	Y	G	G	Y	C	A	C	A	C	A	C	A	C	
Gv×Gl HU1	G	M	R	G	R	Y	Y	M	W	C	Y	G	T	T	K	Y	Y	C	R	Y	Y	R	M	C	Y	T	-	A	C	R	R	C	Y	R	S	S	S	S	S	S	S
Gv×Gl HU2	G	M	R	G	R	Y	Y	M	W	C	Y	R	Y	Y	K	Y	Y	C	R	C	Y	G	M	C	Y	T	-	A	C	G	R	C	Y	R	S	S	S	S	S	S	S
Gv×Gl RS1	G	M	R	G	R	Y	Y	M	T	Y	Y	R	T	T	K	Y	Y	C	R	C	Y	G	M	C	Y	T	-	A	C	G	R	C	Y	R	S	S	S	S	S	S	S
Gv×Gl RS2	K	C	R	R	R	Y	C	M	T	C	T	G	T	W	K	C	Y	C	G	Y	Y	R	M	C	Y	T	-	G	C	G	R	C	Y	R	S	S	S	S	S	S	S
Gv×Gl UA1	G	M	R	G	R	Y	Y	M	W	C	Y	G	T	T	K	Y	Y	C	R	C	Y	R	M	C	Y	T	-	R	Y	G	R	C	Y	R	S	S	S	S	S	S	S
Gv×Gl UA2	G	M	R	G	R	Y	Y	M	W	C	Y	G	Y	Y	K	Y	Y	C	G	C	Y	R	M	C	Y	T	-	R	Y	G	R	Y	Y	R	S	S	S	S	S	S	S
Gv HU	G	C	G	G	G	C	T	C	A	C	C	G	Y	T	G	C	Y	C	A	C	C	G	C	C	C	T	A	C	G	A	C	T	G	G	G	G	G	G	G	G	G
Gv RS	G	C	G	R	G	C	Y	C	W	C	Y	G	T	W	G	C	Y	C	R	C	C	G	C	Y	C	T	A	C	G	A	C	T	G	G	G	G	G	G	G	G	G
Gv UA	G	C	G	G	G	C	T	C	A	C	C	G	T	T	G	C	Y	C	A	C	C	G	C	C	C	T	A	C	G	A	C	T	G	G	G	G	G	G	G	G	G

hybrids consistently displayed additivity at the same position (Table 2), which we accept as direct evidence of hybridity (see also Fuertes Aguilar et al. 1999). Secondly, direct sequence reads obtained using the primer ITS1A (i.e. the ‘forward’ primer) were abruptly unreadable at position 521 in the 3’ direction. The direct reads using primer ITS4 (i.e. the ‘reverse’ primer) revealed the same pattern at the same position in the 5’ direction. Such an abrupt drop in sequence readability usually indicates additivity of paralogous copies of different lengths resulting in ‘misalignment’ (Whittall et al. 2000); this could also be the case here if the additivity consists of nrITS copies of *G. villosa* and *G. linosyris*, which differ in an indel at nt 521. Therefore, this is thought to be true additivity of a diagnostic indel position where the parental species differ in length by 1 bp.

In addition to the biparentally inherited nrITS region, we also sequenced two rapidly evolving plastid regions in order to characterise the variability of the parental species. As plastid DNA is usually transferred only from the maternal parent in plants, these DNA-regions could help us trace the ovule donor of the hybrid samples. The two plastid regions were chosen from the cohort of those plastid IGS regions the taxonomic divergence of which is shallow (usually at the species level) due to their rapid mutation rate. Indeed, the three samples of the two parental species from three geographically distinct regions displayed the same differences between the species (Table 3). *Galatella linosyris* samples had six nt long indels in their *trnH-psbA* sequences (that can be regarded as a mini-satellite repeat of the motif ‘TACTAT’) compared to the *G. villosa* samples. In addition, there was a transition (C/T) in the *trnL-trnF* IGS sequence that mutually separated *G. linosyris* and *G. villosa* (Table 3). Therefore, two clear differences consistently separate these closely related species (see Korolyuk et al. 2015). In addition, we recorded one more difference between the samples: the Hungarian and Ukrainian samples of *G. villosa* had a (T)<sub>8</sub> long microsatellite motif at position 87–93, whereas the rest of the samples of





Fig. 3. – Typical achenes of *Galatella villosa* (A), *G. villosa* × *G. linosyris* (B) and *G. linosyris* (C) (scale bar represents 1 mm).

the parental species had a 7 bp long motif in their *trnH-psbA* sequences. Thus, there is an extra T at position 93 in the above two parental samples (Table 3). As the Serbian *G. villosa* sample did not display this polymorphism, we did not use this position for identifying the maternal lineage of the hybrids because it is inconsistent in samples from the same species. As part of a microsatellite region, this polymorphism is most probably highly variable and can be homoplastic in the species analysed.

Nevertheless, we recorded two species-specific DNA-polymorphisms in the regions analysed, which could be used to trace the maternal parent of the hybrids analysed. Interestingly, the Hungarian and Ukrainian hybrids all displayed the plastid haplotype typical of *G. villosa*, whereas the Serbian hybrid samples shared a haplotype with *G. linosyris* (Table 3).

#### *Hybrid sterility*

All of the achenes of the hybrids were thin and abnormal in shape, compared with those of the parents (Fig. 3). Consequently, we conclude they are infertile. We also compared the pollen of the hybrids and the parental species, because if a hybrid can produce viable pollen, it may still be able to backcross with the parental species. The parental species had regular tricolpate pollen grains with a spherical shape, whereas that of the hybrids was distorted (Electronic Appendix 1).



Table 3. – Polymorphisms displayed between plastid sequences of *Galatella linoisyris* Gl, *G. villosa* Gv, and their hybrids Gv × Gl. Sample provenance is indicated by ISO country codes.

Sample	Nucleotide position in the plastid region		
	<i>trnH-psbA</i>		<i>trnL-trnF</i>
	77–82	94	707
Gl HU	TACTAT	T	T
Gl RS	TACTAT	T	T
Gl UA	TACTAT	-	T
Gv×Gl HU1	-	-	C
Gv×Gl HU2	-	-	C
Gv×Gl RS1	TACTAT	T	T
Gv×Gl RS2	TACTAT	-	T
Gv×Gl UA1	-	-	C
Gv×Gl UA2	-	-	C
Gv HU	-	-	C
Gv RS	-	-	C
Gv UA	-	-	C

### Morphological evidence

The morphological characters measured proved to be useful for separating the taxa studied. The first axis of the LDA (LD1) explained 78.9%, while the second axis (LD2) explained 14.8% of the observed variance. These two axes successfully separate all of the taxa studied (Fig. 4).

The PERMANOVA results were highly congruent with those of the LDA, indicating that the morphology of each pair of taxa differed significantly, but there were no differences between the eastern- and central-European populations of *G. villosa* ( $F = 2.80$ ,  $P = 0.216$ ) and *G. linoisyris* ( $F = 3.50$ ,  $P = 0.450$ ) (Table 4). The leave-one-out cross-validation of the LDA indicates that 100% of the removed specimens were correctly identified for *G. villosa* and *G. subvillosa*, 90.0% for *G. linoisyris* and 94.9% for *G. linoisyris* × *G. villosa*. The largest percentage of misidentifications were recorded for central- and eastern-European populations of *G. linoisyris* and *G. villosa*, but 10% of shoots of *G. linoisyris* were identified as *G. linoisyris* × *G. villosa* and 5.1% of those of the hybrids were identified as *G. ×subvillosa* (Table 5).

The morphological results accord with the molecular data as they clearly show an intermediate position of *G. villosa* × *G. linoisyris* between the parental species and that the *G. villosa* × *G. linoisyris* hybrid is clearly separated from the type specimen of *G. ×subvillosa*.

### Discussion

Based on our molecular genetic results (i.e. additive patterns at variable sites in the nrITS sequences), we are confident that the intermediate plants in our sample are hybrids between the co-occurring species *G. villosa* and *G. linoisyris*. These samples displayed additivity at several other sites, but this simply reflects intraindividual polymorphism within the multicopy nature of the nrITS array (Whittall et al. 2000, Fuertes Aguilar &

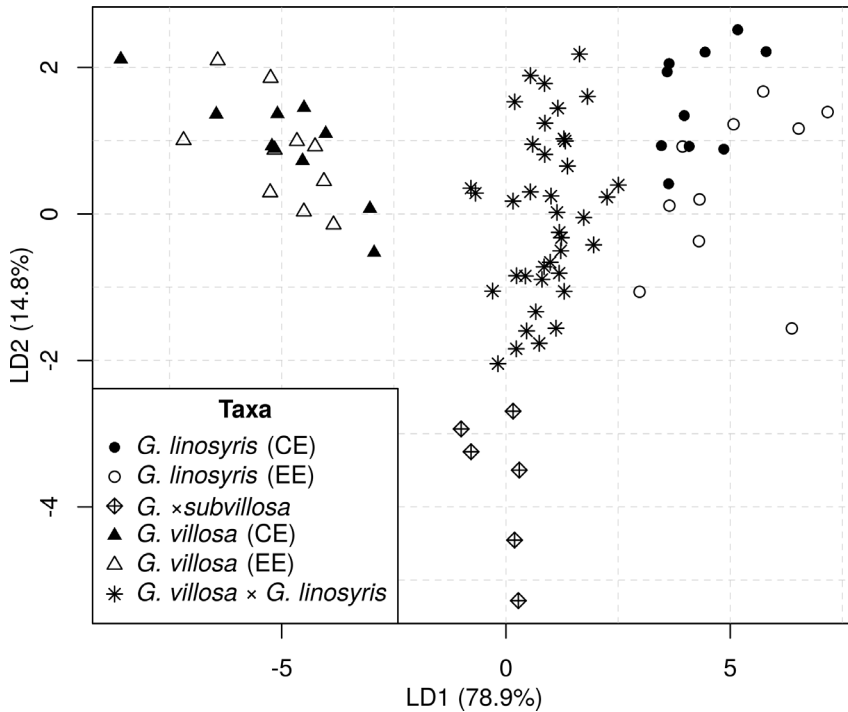


Fig. 4. – Discriminant linear function plot of morphological features studied of the species of *Galatella*.

Table 4. – Results of PERMANOVA showing pairwise morphological differences between the taxa of *Galatella* studied. P-values are shown above the diagonal, whereas F values are given below the diagonal. All P-values were Bonferroni-corrected for multiple testing. Geographic origin of sample groups is indicated as CE for central Europe, and EE for eastern Europe.

Taxon	<i>G. linosyris</i> CE	<i>G. linosyris</i> EE	<i>G. xsubvillosa</i>	<i>G. villosa</i> CE	<i>G. villosa</i> EE	<i>G. villosa</i> × <i>G. linosyris</i>
<i>G. linosyris</i> CE		0.2160	0.0045	0.0015	0.0015	0.0015
<i>G. linosyris</i> EE	2.8		0.0045	0.0015	0.0015	0.0015
<i>G. xsubvillosa</i>	48.2	32.4		0.0030	0.0015	0.0030
<i>G. villosa</i> CE	27.0	29.3	10.4		0.4500	0.0015
<i>G. villosa</i> EE	80.3	71.3	33.0	3.5		0.0015
<i>G. villosa</i> × <i>G. linosyris</i>	32.5	25.3	10.1	23.8	42.8	

Table 5. – Results of the leave-one-out cross-validation in the linear discrimination analyses. All values are proportions. Proportion of adequately categorized removed specimens is shown in the diagonal highlighted in bold. Geographic origin of the groups sampled is indicated as CE for central Europe and EE for eastern Europe.

Taxon	<i>G. linosyris</i> CE	<i>G. linosyris</i> EE	<i>G. xsubvillosa</i>	<i>G. villosa</i> CE	<i>G. villosa</i> EE	<i>G. villosa</i> × <i>G. linosyris</i>
<i>G. linosyris</i> CE	<b>0.8</b>	0.2	0	0	0	0
<i>G. linosyris</i> EE	0.4	<b>0.5</b>	0	0	0	0.1
<i>G. xsubvillosa</i>	0	0	<b>1</b>	0	0	0
<i>G. villosa</i> CE	0	0	0	<b>0.4</b>	0.6	0
<i>G. villosa</i> EE	0	0	0	0.3	<b>0.7</b>	0
<i>G. villosa</i> × <i>G. linosyris</i>	0	0	0.05	0	0	<b>0.95</b>

Nieto Feliner 2003). This is clearly demonstrated by our dataset, where an additive pattern occurred seemingly by chance at other sites than APSs, regardless of the taxon concerned. This result further highlights the importance of making a clear distinction between additive polymorphic sites (Fuertes Aguilar & Nieto Feliner 2003) and simple additivity due to paralogy or the presence of incompletely homogenized sequence variants in nrITS sequences.

Based on the variability in the plastid regions, the maternal parent in the Hungarian and Ukrainian populations can be identified as *G. villosa*, whereas the ovule donor was *G. linoisyris* in the Serbian population. Such bidirectional hybridization may be a sign of the lack of a specific barrier for hybridization in both directions; the ovule donor can be either *G. villosa* or *G. linoisyris*. This may depend on chance events, but clearly, more cases should be examined before drawing a firm conclusion.

Achenes of the hybrid individuals proved to be empty; consequently, these plants can be regarded as primary hybrids incapable of generative reproduction. The distorted shape of the pollen grains also hints at the presence of inviable pollen in the hybrids and thus, the possibility of introgression (i.e. backcrossing) is also unlikely. Nevertheless, we have not examined the viability of these pollen grains, which leaves this to be resolved by further studies.

Using all the morphological characters (habit, shape of leaves and phyllaries), we can conclude that the hybrid described by Tzvelev as *G. ×subvillosa* differs from our hybrid plants of *G. villosa* × *G. linoisyris* in having much shorter leaves, abundant branching in the upper part of stems (with the branches tending to appear arcuate and the synflorescence in the shape of long-branched corymb where the synflorescens is about 30% of the total height of the plant). In the type plants of *G. ×subvillosa*, the villous pubescence on the lower surface of leaves indicates the hybrid influence of *G. villosa*, as suggested by Tzvelev (1994), whereas the abundant, long and partly arcuate branching of the upper stem is indicative of the involvement of *G. divaricata* (Fisch. ex Bieb.) Novopokr. The phyllaries of *G. ×subvillosa* are triangular with acute apices, as in *G. divaricata*, and this could be a reason why Tzvelev erroneously suggested hybridization with *G. linoisyris* (which has narrowly triangular phyllaries and its hybrids also have acute apices of phyllaries).

Considering the shape of the synflorescence, shape of phyllaries, shape and pubescence of leaves, we suggest the hybrid origin of *G. divaricata* × *G. villosa* for the plants described as *G. ×subvillosa*. This hybrid combination has not been previously recognized (cf. Tzvelev 1994). Both presumed parental plants occur in the area where the type was collected (i.e. in the vicinity of the locus classicus) of *G. ×subvillosa* (Elenevsky et al. 2000, 2009), thus allowing for the formation of this hybrid (Fig. 1).

The hybrid corresponding to the formula *G. villosa* × *G. linoisyris* is described as new to science for the first time below.

***Galatella ×feketegaborii*** A. Takács, Sennikov et Sramkó, **nothosp. nova**  
= *Galatella villosa* (L.) Rchb. f. × *Galatella linoisyris* (L.) Rchb. f.

Description: Perennial, usually 30–45 cm tall. Stem erect, leafless below at flowering, puberulent or weakly tomentose. Leaves linear-lanceolate, 10–20× longer than wide, attenuate at base and apex, one-veined, lower ones subglabrous to sparsely tomentose, upper ones puberulent to tomentose, occasionally glandular-punctate above. Capitula

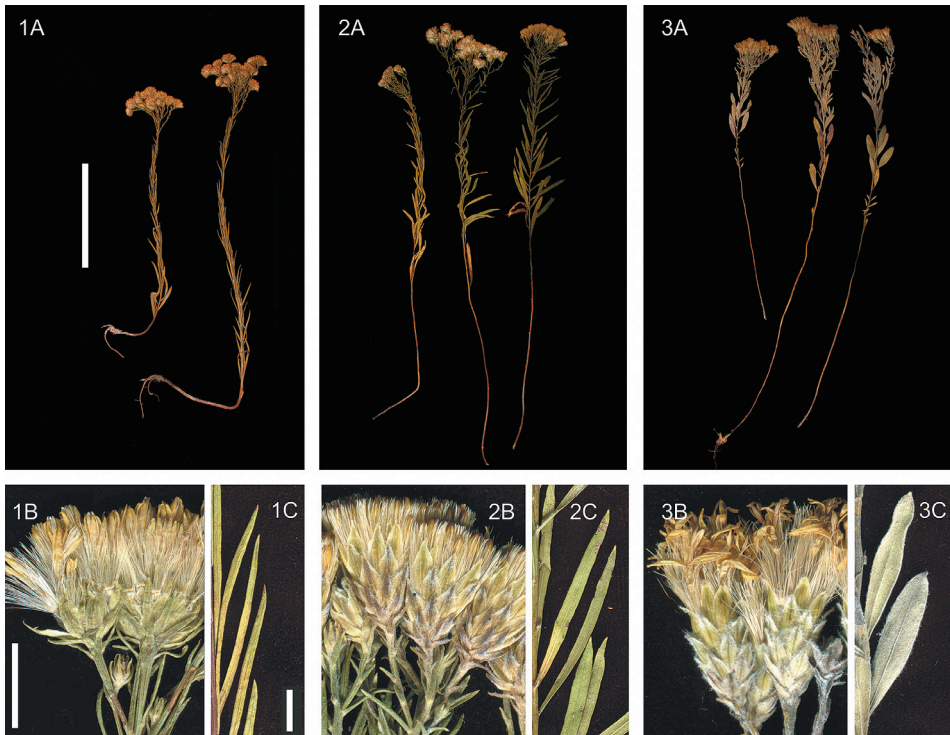


Fig. 5. – Habit (A), capitula (B) and cauline leaves (C) of *Galatella linosyris* (1), *G. villosa* × *G. linosyris* (2) and *G. villosa* (3). Scale bar represents 10 cm (A) and 10 mm (B, C).

narrowly infundibuliform, in dense corymbs. Involucral bracts in several rows, depressed, outer ones tomentose, ovate-lanceolate-triangular, inner ones linear-lanceolate, subglabrous to sparsely tomentose, both with acute apex and scarious, ciliate margins. Tubular flowers 5–15 per capitulum. Pappus hairs pale brownish, 1.5× longer than achene. Achene empty, walls deformed. Pollen grains are not spherical but distorted.

Diagnosis: Intermediate between the parents in terms of the height of the stem, the number and width of cauline leaves (Fig. 5). Unlike in *Galatella villosa*, the stem is taller, with more numerous, narrower and longer leaves with a less developed villous pubescence, and the phyllaries are acute at apex (vs. broadly obtuse). The hybrid also differs from *G. linosyris* in its lower stature, much broader stem leaves with faint villous pubescence below, and triangular (vs. narrowly linear-lanceolate) phyllaries.

Type: Hungary, Borsod-Abaúj-Zemplén county, Tarcál, SW slopes of Tokaj (Kopasz) Hill, 19 October 2013, N 48.1115° E 21.3668°, CEU: 7894.3, coll. A. Takács (holotype: DE-Soo-43283; isotypes: DE-Soo-38311, BP HNHM-TRA00012473) (Fig. 6).

Etymology: The specific epithet commemorates Gábor Fekete (1930–2016), Hungarian botanist-ecologist, who was an honoured researcher of the continental steppes.

Habitat: Dry closed (i.e. with high cover) grasslands, e.g. sloping steppes on stony soils and loess steppes. In Hungary, the habitat of *Galatella* ×*feketegaborii* is secondary grassland on a south-west facing slope, developed on terraces of vineyards abandoned more than 100 years ago. The Serbian population grows in a steppe meadow “island” on





Fig. 6. – Holotype of *Galatella xfeketegaborii* (DE-Soo-43283).

a north-west-facing steep slope surrounded by arable fields. In the Ukraine, it is found on a slope in a valley with steppe vegetation surrounded by arable fields. This place also demonstrates the apparent intermediate ecological conditions this hybrid prefers: the steppe parent (*G. villosa*) grows on west-facing slopes, the forest-steppe parent (*G. linosyris*) on north-facing slopes, whereas the hybrid is confined to the contact zone between that of the parents on the north-west facing parts of slopes.

Distribution: Hungary, Serbia, Ukraine and probably elsewhere where the parental species co-occur (probably present in Bulgaria, Romania, Moldova, Russia and Georgia).

Phenology: The nothospecies flowers during September–October.

Additional specimens examined (paratypes): Hungary, Tarcál, Kiskopasz, 08 September 2017, coll. A. Takács & T. Nagy (DE-Soo-45454). Hungary, Tarcál, Kiskopasz, 04 October 2017, coll. G. Sramkó, T. Zsólyomi, A. Takács (DE-Soo-45455). Serbia, 2,5 km to WNW from vil. Krušce (Крушце), coll. 07 September 2016, A. Molnár V. (DE-Soo-43279). Serbia, Nišava, Krušce, 28 August 2018, coll. A. Takács & L. Laczkó (DE-Soo-46395). Ukraine, Mykolaiv Oblast, Antonivka, Yelanetskyi Step, 01 September 2019, coll. G. Sramkó, A. I. Csathó, L. Bartha L. (DE-Soo-47658; DE-Soo-47659).

See [www.preslia.cz](http://www.preslia.cz) for Electronic Appendix 1

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## Souhrn

Na západním okraji areálu stepního druhu *Galatella villosa* v Maďarsku, Srbsku a na Ukrajině jsme našli rostliny, které byly intermediární mezi tímto druhem a blízce příbuzným *G. linosyris*. S využitím sekvenování biparentálně děděného úseku jaderné DNA (nrITS) jsme prokázali jejich hybridní původ. Porovnáním sekvencí vybraných úseků plastidové DNA (*trnH-psbA* a *trnL-trnF*), která se dědí pouze po mateřské linii, jsme jako mateřský druh identifikovali *G. villosa* u maďarských a ukrajinských kříženců a *G. linosyris* u srbských kříženců. Tyto hybridní rostliny vytvářely pouze sterilní semena. Údajný kříženec těchto dvou druhů byl již v minulosti popsán pod jménem *G. xsubvillosa*. Lineární diskriminační analýza morfologických znaků ale odlišila holotyp jména *G. xsubvillosa* od námi nalezených kříženců *G. linosyris* × *G. villosa*, kteří byli zřetelně intermediární mezi těmito rodičovskými druhy. Oproti původní představě o identitě rodičovských druhů morfologická data dokládají, že na vzniku *G. xsubvillosa* se místo *G. linosyris* podílela *G. divaricata*. Nově nalezené hybridní rostliny *G. linosyris* × *G. villosa* jsme proto popsali jako *G. xfeketegaborii*. Tato studie dokládá možnost využití snadno dostupných molekulárních přístupů k potvrzení hybridního původu rostlin a názorně ukazuje, jak lze aditivní polymorfismus hybridů odlišit od jiných typů intraindividuálního polymorfismu v sekvencích jaderné DNA.

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Appendix 1. – List of herbarium specimens used in the morphometric tests. Geographic origin of the groups sampled is indicated as CE for central Europe and EE for eastern Europe.

*Galatella linosyris* CE – Nine Hungarian specimens and one Romanian specimen from DE without barcode.  
*Galatella linosyris* EE – MW0202840; MW0269795; MW0533461; MW0533465; MW0533492; MW0533493; MW0533494; MW0533498; MW0533502 from Russia.  
*Galatella villosa* CE – BP388371; BP462258; BP462716; BP533803; BP536925; BP538512; BP656677; DE-Soo-38310; DE-Soo-32090 from Hungary and DE-Soo-32092 from Romania.  
*Galatella villosa* EE – MW0533789; MW0533801; MW0533804; MW0533806; MW0533900 from Russia.  
*Galatella* × *subvillosa* – LE01010256 from Russia.