Are there hybrids between *Carex flacca* and *C. tomentosa* in the Czech Republic and Slovakia?

Vyskytují se v České republice a na Slovensku kříženci mezi *Carex flacca* a *C. tomentosa*?

Radomír Ř e p k a, Petra V e s e l á & Jaroslav M r á č e k

Department of Forest Botany, Dendrology and Geobiocenology, Faculty Forestry and Wood Technology, Mendel University, Zemědělská 3, CZ-613 00 Brno, Czech Republic; e-mail: repka@mendelu.cz; petra.vesela@mendelu.cz, mracek@mendelu.cz

Řepka R., Veselá P. & Mráček J. (2014): Are there hybrids between *Carex flacca* and *C. tomentosa* in the Czech Republic and Slovakia? – Preslia 86: 367–379.

At two sites in the Czech Republic and Slovakia we found plants morphologically intermediate between *Carex flacca* subsp. *flacca* and *C. tomentosa.* Here we present the results of morphological and molecular analyses conducted to test whether these plants are the putative hybrid *C.* ×*danielis* (*C. flacca* subsp. *flacca* × *C. tomentosa*). The results revealed a conflict between the morphological characters and molecular markers. Although morphological characters show combinations of characters of the supposed parents and some intermediate characters, molecular markers (ITS, AFLP, *trn*L-F) indicate that the putative hybrid clearly belongs to one of its presumed parents, *C. flacca* subsp. *flacca*. These results refute reports of this hybrid occurring in the Czech Republic and Slovakia.

K e y w o r d s: AFLP, *Cyperaceae*, Czech Republic, *Carex* ×*danielis*, *Carex flacca* subsp. *flacca, Carex tomentosa*, ITS, Slovakia, *trn*L-F

Introduction

The genus *Carex* is taxonomically quite difficult due to the morphological similarities of its representatives, with some taxa having wide intraspecific morphological variability and interspecific hybridization occurring relatively frequently (Stace 1986, Kukkonen & Toivonen 1988). From an historical perspective, there has been an increase in the recognition of *Carex* hybrids because of increased exploration of the genus (Cayouette & Catling 1992). Thus, for Europe, Focke (1881) cites 24 *Carex* hybrids and only ~20 years later, Ascherson & Graebner (1902) 96 hybrids. Kükenthal (1909), in the first world monograph on this genus, includes 141 hybrids, seven of which are newly described. The number of hybrids has continued to increase up to the present, with Koopman (2011) citing 300 *Carex* hybrids in Europe, of which 174 have binomial names and the other 126 are only represented by hybrid formulae. There are a significant number of *Carex* hybrids in the world's floras; however, most of them are concentrated in only a few sections of the genus, primarily sections *Ceratocystis*, *Glareosae*, *Paludosae*, *Phacocystis* and *Vesicariae* (Cayouette & Catling 1992). In the Czech Republic, 29 hybrids are documented by herbarium specimens and another 66 only cited in the literature. The highest numbers of *Carex* hybrids in the Czech flora are found in sections *Ceratocysti*s and *Phacocystis*. Some hybrids in these sections are the most frequent nothospecies in the Czech Republic (*C.* ×*alsatica* Zahn and *C.* ×*turfosa* Fr.; V. Grulich & R. Řepka, unpublished data).

In the present paper, we investigate whether plants that we collected in the Czech Republic and Slovakia that are morphologically intermediate between *C. flacca* and *C. tomentosa* are in fact hybrids of these two species. Only a few publications mention the hybrid *C. flacca* Schreb. × *C. tomentosa* L. *Carex glauca* × *tomentosa* is presented with a question mark in Ascherson & Graebner (1902), who state that Brügger, Kneucker and Kükenthal found this hybrid near Zürich, Karlsruhe and Coburg. Kükenthal (1890) gave the hybrid *C. glauca* × *C. tomentosa* the binomial name *C.* ×*brückneri.* Subsequently, Kükenthal (1909) does not refer to *C.* ×*brückneri* and only states in an endnote to *C. glauca* that the hybrid with *C. tomentosa* is dubious; Koopman (2011), much later, considers *C.* ×*brückneri* to be synonymous with *C. tomentosa.* Léveillé (1912) gave the combination *C. glauca* × *C. tomentosa* the binomial name *C.* ×*danielis*. The protologue for *C.* ×*danielis* H. Lév. is very short and provides only the following information: "vegetative parts like *C. glauca*; bluish perigynia, spikelets and perigynium hairs like *C. tomentosa*; locus classicus Mayenne: Saulges, road to Cossé" (Léveillé 1912). Despite the conciseness of the protologue it appears that the plant described had vegetative organs resembling those of *C. flacca*, but with perigynia and spikelets resembling those of the other parental species, *C. tomentosa*.

More recent European floras or keys either do not mention this hybrid (e.g. Luceño 1994, Egorova 1999, Ciocârlan 2000, Jermy et al. 2007, Fischer et al. 2008, Király 2009) or only repeat Léveillé's description of the hybrid in France. Finally, Koopman (2011) lists *C.* ×*danielis* among accepted nothospecies and presents a distribution map that indicates it occurs in four European countries: France and Belgium (based on Lambignon et al. 1992, 1998), Czech Republic (based on R. Řepka, pers. comm.) and Estonia (based on Kuusk et al. 2003). The existence of the hybrid *C.* ×*danielis* is also mentioned in the manuscript of Vol. 9 of the Flora of the Czech Republic (V. Grulich & R. Řepka, unpublished) and Danihelka et al. (2012) include it in the checklist of the Czech flora.

Classification based only on morphology cannot represent all genetic relationships and similarities (Schmid 1983). Moreover, in the case of *Carex*, morphological studies of the *C. flava* group have revealed variation in morphological characters that cannot be explained exclusively in terms of genetic variation or hybridogenous introgression (Blackstock & Ashton 2010). In addition, some of the characters traditionally used in genus *Carex* may also be environmentally induced (Schmid 1983, Hedrén 1998, Blackstock & Ashton 2010). More generally, in recent decades, the issues of intermediate phenotypes and high phenotypic variability have been subjects of extensive taxonomic discussion (Hedrén 1998, van Droogenbroeck et al. 2006, Lihová et al. 2007, Korpelainen et al. 2010, Jimenés-Mejías et al. 2011, 2014). Therefore, a combination of morphological studies and molecular techniques is a useful way of addressing taxonomic and phylogenetic questions, especially those related to hybridization (Blackstock & Ashton 2010). In vascular plants, molecular markers have proved to be reliable in determining hybrid status, with the AFLP (amplified fragment length polymorphism) technique successfully used to identify hybrid origins of populations in such genera as *Salix* (Beismann et al. 1997), *Aconitum* (Suh et al. 1997), *Magnifera* (Teo et al. 2002) and *Schoenus* (Scotti et al. 2002).

Combinations of different molecular markers, e.g. AFLP, ITS (internal transcribed spacer), cpDNA and mtDNA markers with morphometric or cytological data appear to be especially useful for identifying hybridization and interspecific gene flow (Scotti et al.

2002, Kaplan & Fehrer 2004, 2006, 2009, van Droogenbroeck et al. 2006, Lihová et al. 2007, Kaplan et al. 2009, Galbany-Casals et al. 2012).

In the genus *Carex* molecular markers have been used in some research on hybridization. Based on AFLP data for *Carex* sect. *Phacocystis* Nakamatte & Lye (2007) suggest that *C. bigelowii* subsp. *rigida* W. Schultze-Motel is either a separate species or potential hybrid. A combination of AFLP and restriction-site data revealed that genetic variation in *C. hirsutella* Mack. is relatively high and that it is possibly a consequence of gene flow between this species and one or more other species (Smith & Waterway 2008). In another publication, ITS sequences are used to support the idea that *C. paleacea* Wahlenb. × *C. recta* Boott includes genes from more species than the previously supposed parental species (Korpelainen et al. 2010). Notably, Jiménez-Mejías et al. (2011) reject interspecific hybridization and subsequent clonal reproduction as the source of the individuals they studied from *Carex* sect. *Phacocystis*. Indeed, it is suggested that dissimilarities in morphological and genetic variation e.g. in *Cardamine* polyploids (Lihová et al. 2007) or *Carex* sect. *Phacocystis* (Jiménez-Mejías et al. 2011) need to be reevaluated in the case of taxonomic assignments relying only upon morphological features.

In 2010, we identified a total of 11 plants from two localities that were morphologically intermediate between *C. flacca* and *C. tomentosa*. These plants were morphologically analysed, and showed several intermediate or aberrant (unique) characters. Here we test the hypothesis that plants in the Czech Republic and Slovakia morphologically intermediate between these two *Carex* taxa are the hybrid *C. flacca* × *C. tomentosa* (*C.* cf. × *danielis*). We do this by examining both the morphological evidence for the hybrid status of such specimens and conducting molecular studies to compare the presumed hybrid and parental taxa.

Materials and methods

Material

In total, we studied 12 populations (four *C. flacca,* four *C. tomentosa* and four *C.* cf. ×*danielis*) at two localities in the Czech Republic and Slovakia at which we had collected putative hybrids. The presumed hybrid plants collected at the Czech locality (Nevojice) came from three micropulations (polycormons). We only used 1–11 specimens from each population (see Table 1 and Electronic Appendix 1) as there was little material available. In addition, we incorporated the morphological data of the parental species used in species descriptions of 40–93 specimens collected from the whole of the country in a manuscript submitted to the Flora of the Czech Republic. All the vouchers studied were deposited in the BRNL herbarium unless specified otherwise.

Samples dried using silica gel were used for the molecular analyses (Table 1). To identify species-specific markers, the samples for molecular analyses were selected to detect maximum intraspecific variability; therefore, we used samples not only from the two locations in the Czech Republic and Slovakia but also from geographically independent areas (Bulgaria and Romania). We also successfully used 30-yr old *C.* ×*danielis* herbarium material in the ITS analyses (*C.* cf. ×*danielis*4 – 1984 Sutorý, BRNM, see Table 1).

Table 1. – List of the specimens of *Carex* used in the morphological and molecular analyses. Samples were used for M – morphological analyses, C – chloroplast sequencing, I – ITS sequencing, A – AFLP genotyping.

Taxon	Analysis	Locality
C. flacca subsp. *flacca* (1a)	M C I A	CZ, southern Moravia, Nevojice, subxerophilous grassy slope at end of valley about 2.5 km N of the village, 294 m a.s.l.; GPS 49°08'54.2" N, 17°02'08.4" E
C. flacca subsp. *flacca* (1b)	M	CZ, southern Moravia, Nevojice, subxerophilous grassy slope at end of valley about 2.5 km N of the village, 294 m a.s.l.; GPS 49°08'54.8" N, 17°02'09.0" E
C. flacca subsp. *flacca* (1c)	M	CZ, southern Moravia, Nevojice, grassy slope at end of valley about 2.5 km N of the village, 300 m a.s.l. (1984 Sutorý, BRNM)
C. flacca subsp. *flacca* (2)	A	CZ, White Carpathians, Velká nad Veličkou, subxerophilous grassland in Zahrady pod Hájem Nature Reserve, E of the village, 356 m a.s.l. GPS 48°53'23.0" N, 17°31'84.0" E
C. flacca subsp. *flacca* (3)	M C I A	SK, White Carpathians, Chvojnica – Martišovci settlement, moist spring area directly above the uppermost house in the settlement, 440 m a.s.l., GPS 48°47'23.7" N, 17°24'23.7" E
C. flacca subsp. *flacca* (4)	A	CZ, southern Moravia, Brno-Líšeň, on bank of reservoir in Mariánské údolí valley, 2.3 km NE of the town hall (2006 Hralová & Bureš, BRNU), 250 m a.s.l., GPS 49° 13'14.8" N, 16° 42'49.4" E
C. flacca subsp. *erythrostachys*	A	BG, Varna, Emona (near Cape Emine), undergrowth of thermophilic oak forest above sandy beach, Irakli, 2.5 km N of the cape, 8 m a.s.l., GPS 42°44'60.8" N, 27°53'42.0" E
C. tomentosa (1a)	M C I A	CZ, southern Moravia, Nevojice, subxerophilous grassy slope at end of valley about 2.5 km N of the village, 294 m a.s.l., GPS 49°08'54.2" N, 17°02'08.4" E
C. tomentosa (1b)	M	CZ, southern Moravia, Nevojice, subxerophilous grassy slope at end of valley about 2.5 km N of the village, 294 m a.s.l., GPS 49°08'54.8" N, 17°02'09.0" E
C. tomentosa (1c)	M	CZ, South Moravia, Nevojice, grassy slope at end of valley about 2.5 km N of the village, 300 m a.s.l., (1984 Sutorý, BRNM)
C. tomentosa (2)	M C I A	SK, White Carpathians, Chvojnica – settlement named Martišovci, moist spring area directly above the upper house in the settlement, 440 m a.s.l., GPS 48°47'23.7" N, 17°24'23.7" E
C. tomentosa (3)	A	ROM, Banat, Sfanta Elena, *Quercus cerris* forest, 300 m from the village, 355 m a.s.l., GPS 44°40'89.0" N, 21°22'39.0" E
C. cf. ×*danielis* (1)	M C I A	CZ, southern Moravia, Nevojice, subxerophilous grassy slope at end of valley N of the village, first population, 298 m a.s.l., GPS 49°08'54.2" N, 17°02'08.4" E
C. cf. ×*danielis* (2)	M C I A	CZ, southern Moravia, Nevojice, subxerophilous grassy slope at end of valley N of the village, second population, 50 m from (1), 292 m a.s.l., GPS 49°08'54.8" N, 17°02'09.0" E
C. cf. ×*danielis* (3)	M C I A	SK, White Carpathians, Chvojnica – Martišovci settlement, damp spring area, directly above the uppermost house in the settlement, 440 m a.s.l., GPS 48°47'23.7" N, 17°24'23.7" E
C. cf. ×*danielis* (4)	M I	CZ, southern Moravia, Nevojice, grassy slope at end of valley about 2.5 km N of the village, 300 m a.s.l. (1984 Sutorý, BRNM)

Morphological study

The herbarium specimens of the presumed hybrid and its parent species, *C. flacca* and *C. tomentosa*, were used to measure 12 quantitative features (stem height and diameter, length and width of leaves, length of inflorescence, length of lower bract, length and width of male spike, length and width of female spike, number of female spikes, length of peduncle of female spike, length of female glume, length and width of perigynium, and length of perigynium beak) and 10 qualitative features (type of rhizome, leaf colouring, male spike: shape and colour, female spike position, perigynium shape and colour, perigynium hairs, perigynium beak features, fertility). The plants from populations labelled as "presumed hybrid" were preliminarily identified as *C.* cf. ×*danielis* based on the following characters: shape and position of female spikes, length of female spike peduncle, size of perigynium, type of perigynium hairs and fertility. The material was studied using a ruler and binocular microscope.

Molecular analysis

DNA was isolated using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. PCR was performed using MyTaq polymerase (Bioline) and primers ITS3i (5'-GCATCGATGAAGAACGTAGC-3') and ITS4i (5'-GGTAGTCCCGCCTGACCTGG-3') (ITS2 region) with the following reaction conditions: 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 50 s. Subsequent sequencing on a ABI 3730xl DNA Analyser (Applied Biosystems, Forster City, USA) without PCR sub cloning was used to detect both genotypes of the parental species by presence of double or multiple signals at polymorphic bases in samples of the presumed hybrids. The *trn*L-F region was amplified using primers c and f (Taberlet et al. 1991). The modified PCR conditions are those previously described by Řepka & Mráček (2012). DNA sequence alignments were performed in Bioedit version 7.0.9.0 (Hall 1999).

The AFLP technique was performed according to Vos et al. (1995) with minor modifications. The first reaction, comprising restriction and ligation, was performed with 200 ng of DNA. The preselective amplification used the primers EcoRI-A/MseI-C. Selective amplification was performed using EcoRI-ACG-FAM/Msel-CGG, EcoRI-ACG-NED/Msel-CCA, EcoRI-ATT-FAM/Msel-CGA and EcoRI-ATT-FAM/Msel-CGG primer combinations. Fragments were separated on an ABI PRISM 3100 Avant Genetic Analyser and ABI 3730xl DNA Analyser using the GS-500 LIZ size standard. Fragments were analysed using GeneMapper v. 4.1 (Applied Biosystems, Forster City, USA). AFLP markers were scored in the range of 50–500 bp. A matrix (1 = present; 0 = absent) was created in which only well-separated fragments were scored. Reproducibility of AFLP data was determined using independently analysed redundant samples. Species-specific markers for both taxa were selected based on the following criteria: species-specific markers were fragments present in all samples of a respective parental species and absent in those of the other species. To analyse molecular variation in samples of *Carex*, principal component analyses (PCA) was applied to AFLP data, using the program PAST ver. 3.02a (Hammer et al. 2001).

Results

Morphology

The plant found at Chvojnica (*C.* ×*danielis*3) had characters indicating it was intermediate in its morphology between *C. flacca* subsp. *flacca* and *C. tomentosa*. The vegetative organs (slenderness of plant and gracility of these organs) and quantitative characteristics of the reproductive organs generally placed the plant between the assumed parent species, but some of them, e.g. short upright spikelets in short inflorescence, short peduncles and small perigynium, placed the potential hybrid plant at the edge of the variation of *C. flacca* subsp. *flacca* (hereinafter referred to as *C. flacca*) (Electronic Appendix 1). Moreover, the surfaces of the perigynia were covered with papillae, as is common in *C. flacca*. Achenes in the perigynia were fully developed.

Most of the presumed hybrid plants from Nevojice had vegetative characters resembling those of one of the parents (*C. flacca*), but differed from it mainly in terms of the characteristics of the reproductive organs. The herbarium material collected by K. Sutorý in 1984 (*C.* ×*danielis*4) includes plants that had narrow leaves, short inflorescences with short bracts, shortened spikelets on erect upright peduncles, short perigynia, obovate and more bloated with relatively dense covering of papillae, all suggestive of hybrid status, but in other characters matched the presumed parental species *C. flacca*. One of these plants was sterile and the surface of the perigynium was densely covered with wide-based hairs. In this character it resembled *C. tomentosa*, which has perigynia that are commonly very thickly covered with long whitish yellow hairs, which in some specimens are wider and flatter at the base; their tips are straight or turned upwards. In contrast, *C. flacca* plants have perigynia covered with separate white erect hairs or, in some specimens, much smaller dense papillae on the upper half. Also, perigynia of *C. flacca* commonly have papillae, both on the surface and at the edges; the surface is glossy or matt. However, some also have finger-shaped hairs without a wide base, only 0.1 mm long, and are minutely papillose or scabrid (as obvious from our extensive studies of Czech and Slovak herbarium material of this species).

One micropopulation at Nevojice (*C.* cf. ×*danielis*1) had rhizomes very similar to those of *C. tomentosa*, although the generative organs corresponded mainly to those of *C. flacca*. These plants also had much shorter, upright female spikelets on short peduncles, with maturing achenes. This micropopulation contained a single sterile plant; it also included a plant that had perigynium beaks resembling those of *C. tomentosa*. Another Nevojice micropopulation (*C.* ×*danielis*2) was intermediate in having a short inflorescence and short erect female spikelets on short peduncles and bracts shorter than the inflorescences. The hybrid origin of the material from this micropopulation was, however, also supported by a single sterile plant.

Besides other intermediate features and plant sterility, the type and density of the hairs on the perigynium were the most significant features suggesting that these plants were hybrids (see Electronic Appendix 1).

Molecular markers

The results of the analysis of the *trn*L-F region (Fig. 1) clearly identified *C. flacca* as the maternal species, because in angiosperms the chloroplast genome is inherited maternally

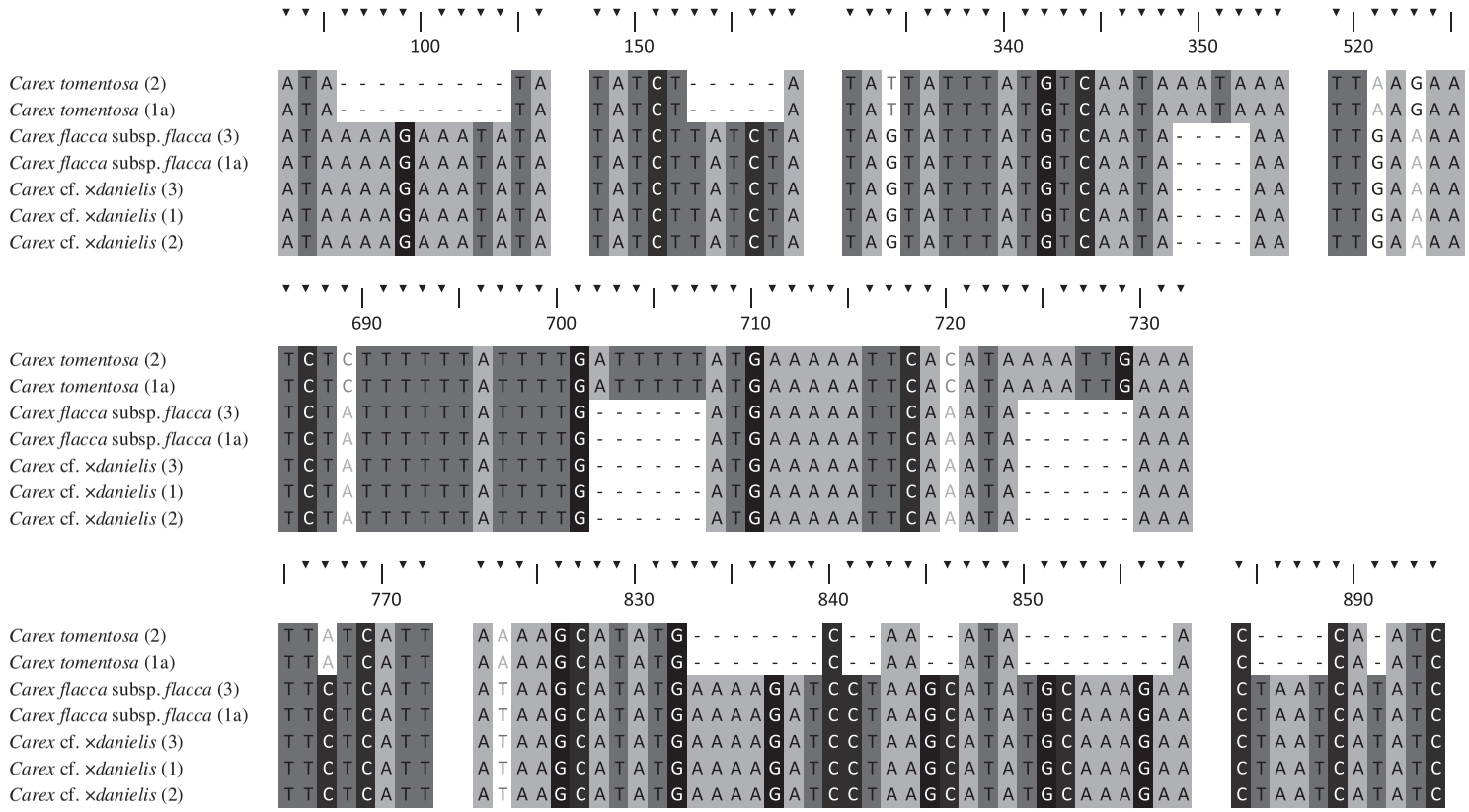

Fig. 1. – Polymorphic regions in the DNA alignment of the chloroplast encoded region *trn*L-F of the *Carex* taxa studied.

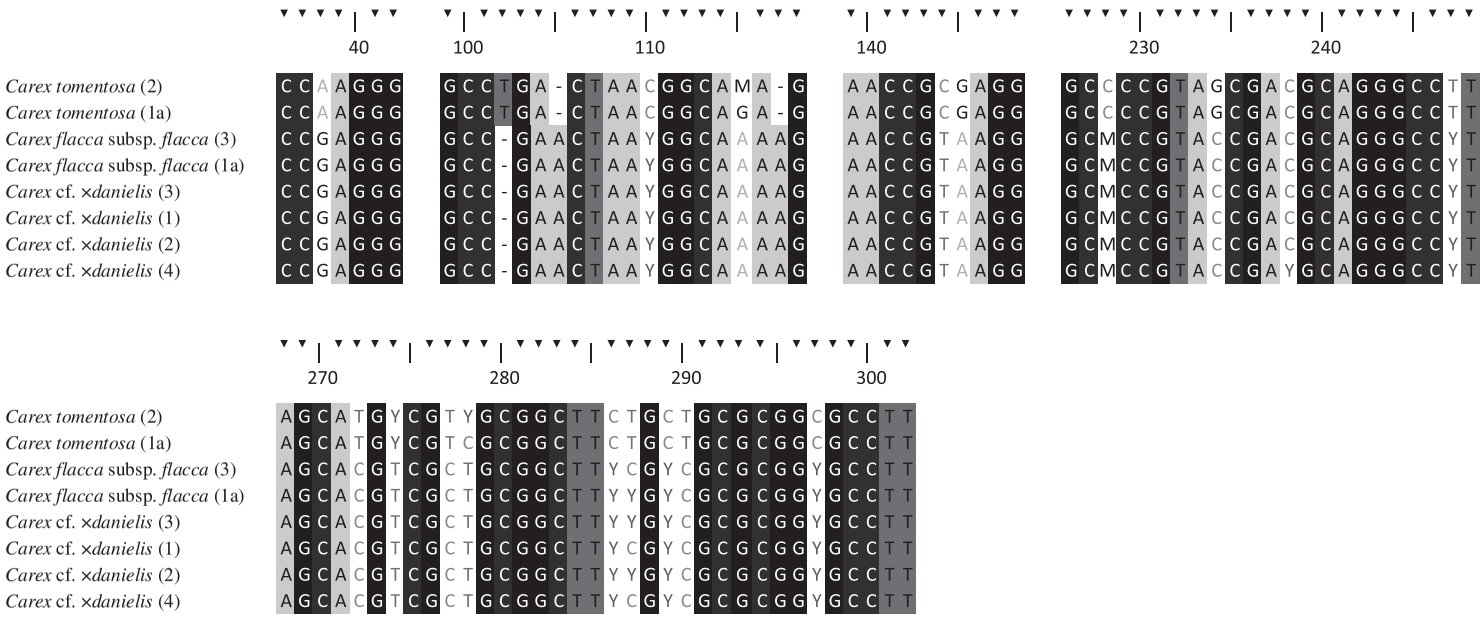

Fig. 2. – Polymorphic regions in the DNA alignment of the nuclear encoded ITS2 spacer in the *Carex* taxa studied.

Carex tomentosa (1a)	0	0	1	0	0	1	0	1	1	1	1	0	0	0	0	1	1	0	1	0	1	0	1	0	1	0	0	0	0	1
Carex tomentosa (2)	0	0	1	0	0	1	0	1	1	1	1	0	0	0	0	1	1	0	1	0	1	0	1	0	1	0	0	0	0	1
Carex tomentosa (3)	0	0	1	0	0	1	0	1	1	1	1	0	0	0	0	1	1	0	1	0	1	0	1	0	1	0	0	0	0	1
Carex flacca subsp. *flacca* (1a)	1	1	0	1	1	0	1	0	0	0	0	1	1	1	1	0	0	1	0	1	0	1	0	1	0	1	1	1	1	0
Carex flacca subsp. *flacca* (2)	1	1	0	1	1	0	1	0	0	0	0	1	1	1	1	0	0	1	0	1	0	1	0	1	0	1	1	1	1	0
Carex flacca subsp. *flacca* (3)	1	1	0	1	1	0	1	0	0	0	0	1	1	1	1	0	0	1	0	1	0	1	0	1	0	1	1	1	1	0
Carex flacca subsp. *flacca* (4)	1	1	0	1	1	0	1	0	0	0	0	1	1	1	1	0	0	1	0	1	0	1	0	1	0	1	1	1	1	0
Carex flacca subsp. *erythrostachys*	1	1	0	1	1	0	1	0	0	0	0	1	1	1	1	0	0	1	0	1	0	1	0	1	0	1	1	1	1	0
Carex cf. ×*danielis* (3)	1	1	0	1	1	0	1	0	0	0	0	1	1	1	1	0	0	1	0	1	0	1	0	1	0	1	1	1	1	0
Carex cf. ×*danielis* (1)	1	1	0	1	1	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	0	1	0	1	0	1	1	1	1	0
Carex cf. ×*danielis* (2)	1	1	0	1	1	0	1	0	0	0	0	1	1	0	1	0	0	1	0	1	0	1	0	1	0	1	1	1	1	0

Fig. 3. – Species-specific AFLP markers for *Carex flacca* and *C. tomentosa* and their presumed hybrid.

(Rebound & Zeyl 1994). The ITS region is a nuclear multicopy region that is inherited biparentally. The alignment of the ITS2 region (Fig. 2) revealed only the genotype of *C. flacca* in samples of the presumed hybrid (*C.* cf. ×*danielis*).

The AFLP data were obtained using four primer combinations. The use of 33 redundant samples indicated the reproducibility was 99.3%. From 241 scored fragments we selected 30 species-specific loci, fragments of which were present in all samples of one of the parent species, but absent in the other. The same genotype as *C. flacca* was found at all loci of one sample of the presumed hybrid taxon (*C.* cf. ×*danielis*) and in 29 of 30 loci of another two samples (Fig. 3). The PCA plot includes the presumed hybrid in the group of *C. flacca* samples, with the samples of *C. tomentosa* placed in a distinct group (Fig. 4). The first and second coordinates of the PCA explain 39% and 13% of the variation in the AFLP dataset, respectively. We may conclude that the AFLP data do not support the hypothesis of a hybrid origin of the samples identified as the presumed hybrid (*C.* cf. ×*danielis*).

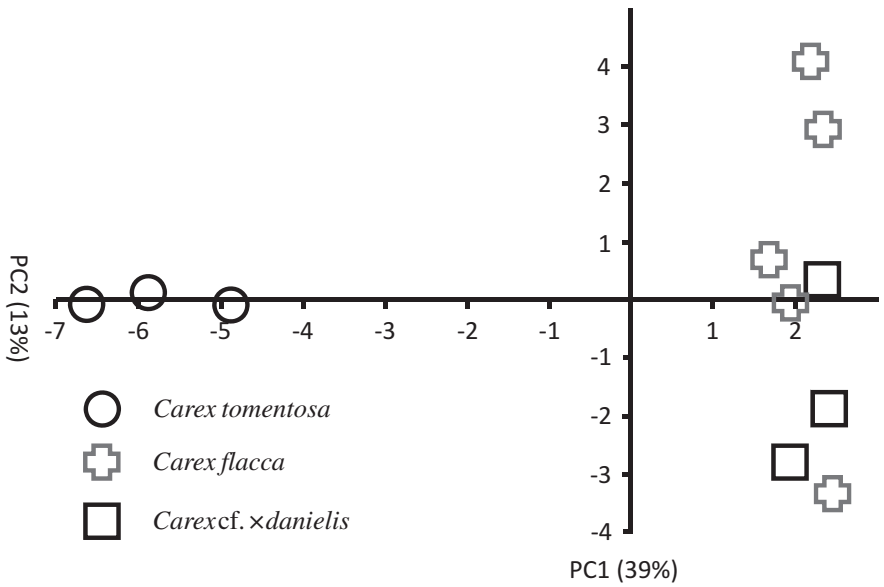

Fig. 4. – PCA plot of *Carex flacca* and *C. tomentosa* individuals and their presumed hybrid.

Discussion

The morphological and molecular analyses provided very different answers regarding the status of the presumed hybrid *C.* cf. ×*danielis*. The morphological analysis indicated that samples of the assumed hybrid *C.* cf. ×*danielis* have combinations of characters of the supposed parents as well as some intermediate characters. Besides the previously mentioned sterility of some plants, the hairs on the perigynium of one plant from the Nevojice site (described above) showed an influence of *C. tomentosa*, even though the vegetative character of *C. flacca* predominated in these plants. Also the fertility of plants, as recorded for the material from Chvojnica, cannot be used as an argument to reject the hybrid origin of this plant material (Galbany-Casals et al. 2012), because in some *Carex* sections (e.g. *Phacocystis*) there are partially or completely fertile hybrids (Faulkner 1972, Jermy et al. 2007). However, contrary to the morphological analysis, the molecular evidence fully supports the conclusion that the material belongs to *C. flacca*. AFLP analyses provided 30 species-specific fragments, which indicated that the samples of *C.* cf. ×*danielis* have a pattern identical or in two samples nearly identical to *C. flacca* (Fig. 3). The variability is for one locus per sample, which can be explained as intraspecific variability previously undetected in *C. flacca* due to the limited number of samples and collection sites. Moreover, the PCA plot (Fig. 4) of AFLP genetic variation shows no differentiation between the *C.* cf. ×*danielis* and *C. flacca* samples.

Other studies of putative hybridization in the genus *Carex* have shown both agreement and disagreement between morphological and genetic-marker analyses. Thus, for one population of the *C. flava* group (Blackstock & Ashton 2010), the two approaches produced similar results, whereas in another two they were not similar or enigmatic. Our results, refuting hybridization and documenting higher morphological variation in *C. flacca*, are similar to those for *Carex* sect. *Phacocystis* (Jiménez-Mejías et al. 2011). Indeed, these authors suggest that the low contribution of hybridization to overall genetic variation means that the frequency of hybridization has been overestimated. They thus explain the previously reported putative hybrid zones of members of *Carex* sect. *Phacocystis* as representing wider than expected morphological variation rather than a result of hybridization (Jiménez-Mejías et al. 2011).

Based on our results, the reported occurrences of the hybrid *C.* cf. ×*danielis* in the Czech Republic (see Danihelka et al. 2012) and western Slovakia are refuted, as the plants originally identified as *C.* cf. ×*danielis* in fact belong to *C. flacca.* This is not to say, however, that it is impossible for the putative parental species *C. flacca* and *C. tomentosa* to have hybridized anywhere in Europe. In fact, both of these species occur in the same habitats, sub-Atlantic and sub-continental broad-leaved dry grasslands (alliances *Bromion erecti* and *Cirsio-Brachypodion*) growing on heavy calcareous tertiary sediments (Novák & Chytrý 2007), and the two taxa meet across a wide area from Great Britain to eastern and south-eastern Europe (see Meusel et al. 1965). They not only overlap broadly in terms of geographical distribution and habitat, but also phenologically, which creates opportunities for hybridization. Moreover, although *C. flacca* and *C. tomentosa* are phylogenetically rather distant, as shown by differences in chromosome numbers (76 and 48, respectively; Rotreklová et al. 2011) and genome size (1.06 and 0.44 pg, respectively; Lipnerová et al. 2013), hybridization in the subgenus *Carex* has occurred between sections recognized as quite distant based on both morphology (Egorova 1999) and molecular data

(Hendrichs et al. 2004). Importantly, we have not genetically analysed any putative *C.* cf. ×*danielis* from sites outside the Czech Republic and Slovakia. Nevertheless, the results based on our samples raise the question of whether other putative specimens of this hybrid from elsewhere are indeed hybrids.

Our study reaffirms the importance of the conclusions of Lihová et al. (2007) that relying only on morphological characters can be misleading and insufficient for the identification of hybrids, for which it is necessary to use modern molecular methods (i.e. markers). Our results also demonstrate that the morphological variability of putative parents (in our case *C. flacca*) is often insufficiently studied and the variation of taxonomically important characters poorly described. This can lead to specimens being incorrectly designated as products of hybridization when characters of these plants fall outside the described variation. Our findings also suggest that the frequency of hybrids in *Carex* can be much smaller than is generally recognized, with further research needed to reveal how many of the ~300 European hybrids reported by Koopman (2011) are genuine hybrids. Verification of hybridization events and genetic introgressions using multiple approaches are important for a better explanation of the morphological and genetic variation in the genus *Carex.*

See www.preslia.cz for Electronic Appendix 1

Acknowledgements

We would like to thank GAČR project no. 206/09/1405 for financially supporting this study. We thank K. Sutorý (BRNM) for providing herbarium material of putative hybrids and their parental species and for help in the field. We also thank two anonymous reviewers for valuable comments on the first version of the manuscript. We are indebted to J. Rosenthal for his linguistic help and critical reading of the manuscript. Tony Dixon kindly improved English of the accepted manuscript.

Souhrn

Na jedné lokalitě v České republice a na jedné na Slovensku byly nalezeny přechodné morfotypy mezi druhy *Carex flacca* a *C. tomentosa*. Po analýze morfologických znaků (12 kvantitativních a 10 kvalitativních) byly tyto rostliny určeny jako pravděpodobný hybrid *C.* ×*danielis* Léveillé, takto publikovány v checklistu flóry ČR a uvedeny s popisem v rukopisu 9. dílu Květeny ČR. V tomto článku jsou prezentovány protichůdné výsledky z morfologické i molekulární analýzy tohoto rostlinného materiálu z obou lokalit. Ač některé morfologické znaky, především na generativních orgánech, ukazují na intermediaritu (nebo výjimečně i unikátnost znaku) mezi oběma druhy, použité molekulární markery (ITS, AFLP, *trn*L-F) jasně dosvědčují, že materiál z obou lokalit patří k jednomu z předpokládaných rodičovských druhů, *C. flacca*. Na základě našich výsledků musíme křížence *C.* ×*danielis* z checklistu ČR odstranit a podobně vyloučit jeho existenci na dosud jediné známé lokalitě na Slovensku. Tyto výsledky mají dopad pouze na populace dvou lokalit v obou státech a neříkají zatím nic o tom, zda tento hybrid je vůbec v přírodě reálný a zda jsou údaje o jeho existenci na území některých evropských států relevantní.

References

Ascherson P. & Graebner P. (1902): Synopsis der mitteleuropäischen Flora. Vol. 2/2. – H. Stürtz, Leipzig.

Beismann H., Barker J. H. A., Karp A. & Speck T. (1997): AFLP analysis sheds light on distribution of two *Salix* species and their hybrid along a natural gradient. – Mol. Ecol. 6: 989–993.

Blackstock N. & Ashton P. A. (2010): Genetic markers and morphometric analysis reveal past hybridization and introgression in putative *Carex flava* L. s. str. (*Cyperaceae*) hybrid populations. – Plant Syst. Evol. 287: 37–47.

Cayouette J. & Catling P. M. (1992): Hybridization in the genus *Carex* with special reference to North America. – Bot. Rev. 58: 351–438.

Ciocârlan V. (2000): Flora ilustrată a României. *Pteridophyta* et *Spermatophyta* [Illustrated flora of Romania. *Pteridophyta* and *Spermatophyta*]. – Editura Ceres, Bucuresti.

Danihelka J., Chrtek J. & Kaplan Z. (2012): Checklist of vascular plants of the Czech Republic. – Preslia 84: 647–811.

Egorova T. V. (1999): Osoki (*Carex* L.) Rossii i sopredelnych gosudarstv (v predelach byvšego SSSR) [The sedges (*Carex* L.) of Russia and adjacent states (within the limits of the former USSR)]. – Sankt-Peterburgskaja Gosudarstvennaja Chimiko-farmacevticeskaja Akademija, Sankt-Peterburg & Missouri Botanical Garden Press, St. Louis.

Faulkner J. S. (1972): Chromosome studies on *Carex* section *Acutae* in NW Europe. – Bot. J. Linn. Soc. 65: 271–301.

Fischer M. A. (ed.) (2008): Exkursionsflora von Österreich. – Eugen Ulmer, Stuttgart & Wien.

Focke W. O. (1881): Die Pflanzen-mischlinge. – G. Borntraeger, Berlin.

Galbany-Casals M., Carnicero-Campmany P., Blanco-Moreno J. M. & Smissen R. D. (2012): Morphological and genetic evidence of contemporary intersectional hybridization in mediterranean *Helichrysum* (*Asteraceae, Gnaphalieae*). – Plant Biol. 14: 789–800.

Hall T. A. (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. – Nucleic Acids Symposium Series 41: 95–98.

Hammer Ø., Harper D. A. T. & Ryan P. D. (2001): PAST: paleontological statistics software package for education and data analysis. – Palaeontol. Electron. 4: 1–9.

Hedrén M. (1998): Status of *Carex bergrothii* (*Cyperaceae*) on Gotland, SE Sweden. – Nordic J. Bot. 18: 41–49.

Hendrichs M., Oberwinkler F., Begerow D. & Bauer R. (2004): *Carex*, subgenus *Carex* (*Cyperaceae*): a phylogenetic approach using ITS sequences. – Plant Syst. Evol. 246: 89–107.

Jermy A. C., Simpson D. A., Foley M. J. Y. & Porter M. S. (2007): Sedges of the British Isles. Ed. 3. – Botanical Society of the British Isles, London.

Jiménez-Mejías P., Escudero M., Guerra-Cárdenas S., Lye K. A. & Luceño M. (2011): Taxonomic delimitation and drivers of speciation in the Ibero-North African *Carex* sect. *Phacocystis* river-shore group (*Cyperaceae*). – Am. J. Bot. 98: 1855–1867.

Jiménez-Mejías P., Luceño M. & Martin-Bravo S. (2014): Species boundaries within the Southwest Old World populations of the *Carex flava* group. – Syst. Bot. 39: 117–131.

Kaplan Z. & Fehrer J. (2004): Evidence for the hybrid origin of *Potamogeton* ×*cooperi* (*Potamogetonaceae*): traditional morphology-based taxonomy and molecular techniques in concert. – Fol. Geobot. 39: 431–453.

Kaplan Z. & Fehrer J. (2006): Comparison of natural and artificial hybridization in *Potamogeton*. – Preslia 78: 303–316.

Kaplan Z. & Fehrer J. (2009): An orphaned clone of *Potamogeton* ×*schreberi* in the Czech Republic. – Preslia 81: 387–397.

Kaplan Z., Fehrer J. & Hellquist C. B. (2009): New hybrid combinations revealed by molecular analysis: the unknown side of North American pondweed diversity (*Potamogeton*). – Syst. Bot. 34: 625–642.

Király G. (ed.) (2009): Új magyar füvészkönyv. Magyarország hajtásos növényei [New Hungarian herbal. Vascular plants of Hungary. Identification key]. – Aggteleki Nemzeti Park Igazgatóság, Jósvafö.

Koopman J. (2011): *Carex* Europaea. The genus *Carex* L. (*Cyperaceae*) in Europe, 1. – Margraf Publishers, Weikersheim.

Korpelainen H., Virtanen V., Kostamo K. & Väre H. (2010): Hybridization and introgression in *Carex aquatilis* and *C. paleacea*. – Plant Syst. Evol. 287: 141–151.

Kükenthal G. (1890): *Carex glauca* × *tomentosa* n. hybr. = *C. Brückneri* m. – Deutsche Bot. Monatsschr. 8: 107–108.

Kükenthal G. (1909): *Cyperaceae* – *Caricoideae*. – In: Engler A. (ed.), Das Pflanzenreich 38: 1–824, W. Engelmann, Leipzig.

Kukkonen I. & Toivonen H. (1988): Taxonomy of wetland carices. – Aquat. Bot. 30: 5–22.

Kuusk V., Tabaka L. & Jankevičiene R. (eds) (2003): Flora of the Baltic countries. Compendium of vascular plants. – Eesti Loodusfoto AS, Tartu.

Lambignon J., De Langhe J.-E., Delvosalle L. & Duvigneaud J. (1992): Nouvelle flore de la Belgique, de Grand–Duché de Luxembourg, du nord de la France et des Régions Voisines. Ed. 4. – Patrimoine du Jardin botanique national de Belgique, Meise.

Lambignon J., De Langhe J.-E., Delvosalle L. & Duvigneaud J. (1998): Nouvelle flore de la Belgique, de Grand–Duché de Luxembourg, du nord de la France et des Régions Voisines. Ed. 5. – Patrimoine du Jardin botanique national de Belgique, Meise.

Léveillé A. A. H. (1912): *Carex* ×*danielis*. – Bull. Geogr. Bot. 22: 48.

Lihová J., Kučera J., Perný M. & Marhold K. (2007): Hybridization between two polyploid *Cardamine* (*Brassicaceae*) species in North-western Spain: discordance between morphological and genetic variation patterns. – Ann. Bot. 99: 1083–1096.

Lipnerová I., Bureš P., Horová L. & Šmarda P. (2013): Evolution of genome size in *Carex* (*Cyperaceae*) in relation to chromosome number and genomic base composition. – Ann. Bot. 111: 79–94.

Luceño M. (1994): Monografía del genéro *Carex* en la Península Ibérica e Islas Baleares. – Ruizía 14: 5–139.

Meusel H., Jäger E. & Weinert E. (1965): Vergleichende Chorologie der Zentraleuropäischen Flora, Vol. 1. – Gustav Fischer Verlag, Jena.

Nakamatte E. & Lye K. A. (2007): AFLP-based differentiation in north Atlantic species of *Carex* sect. *Phacocystis*. – Nord. J. Bot. 25: 318–328.

Novák J. & Chytrý M. (2007): *Cirsio-Brachypodion pinnati*, *Bromion erecti*. – In: Chytrý M. (ed.), Vegetation of the Czech Republic 1: 425–449, Academia, Praha.

Reboud X. & Zeyl C. (1994): Organelle inheritance in plants. – Heredity 72: 132–140.

Řepka R. & Mráček J. (2012): *Gymnocalycium esperanzae*: a nothospecies? – Haseltonia 18: 105–115.

Rotreklová O., Bureš P., Řepka R., Grulich V., Šmarda P., Hralová I., Zedek F. & Koutecký T. (2011): Chromosome numbers of *Carex*. – Preslia 83: 25–58.

Schmid B. (1983): Notes on the nomenclature and taxonomy of the *Carex flava* group in Europe. –Watsonia 14: 309–319.

Scotti I., Mariani A., Verona V., Candolini A., Cenci C. A. & Olivieri A. M. (2002): AFLP markers and cytotaxonomic analysis reveal hybridization in the genus *Schoenus* (*Cyperaceae*). – Genome 45: 222–228.

Smith T. W. & Waterway M. J. (2008): Evaluating species limits and hybridization in the *Carex complanata* complex using morphology, amplified fragment length polymorphisms, and restriction fragment analysis. – Botany 86: 809–826.

Stace C. A. (1986): Hybridization and plant taxonomy. – Symb. Bot. Ups. 27: 9–18.

Suh Y., Kim S. & Park C. W. (1997): AFLP examination for putative hybrids between *Aconitum japonicum* ssp. *napiforme* and *A. jaluense* (*Ranunculaceae*). – Korean J. Plant Taxon. 27: 59–71.

Taberlet P., Gielly L. & Bouvet J. (1991): Universal primers for amplification of three non-coding regions of chloroplast DNA. – Plant Mol. Biol. 17: 1105–1109.

Teo L. L., Kiew R., Set O., Lee S. K. & Gan Y. Y. (2002): Hybrid status of kuwini, *Mangifera odorata* Griff. (*Anacardiaceae*) verified by amplified grafment length polymorphism. – Mol. Ecol. 11: 1465–1469.

van Droogenbroeck B., Kyndt T., Romeijn-Peeters E., van Thuyne W., Goetghebeur P., Romero-Motochi J. P. & Gheysen G. (2006): Evidence of natural hybridization and introgression between *Vasconcellea* species (*Caricaceae*) from southern Ecuador revealed by chloroplast, mitochondrial and nuclear DNA markers. – Ann. Bot. 97: 793–805.

Vos P., Hogers R., Bleeker M., Reijans M., van de Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M. & Zabeau M. (1995): AFLP: a new technique for DNA fingerprinting. – Nucleic Acids Res. 23: 4407–4414.

Received 23 April 2014
Revision received 10 October 2014
Accepted 17 October 2014