Preslia 94: 183–213, 2022
doi: 10.23855/preslia.2022.183

Preslia

Relative DNA content differences reliably identify *Solidago ×niederederi*, a hybrid between native and invasive alien species

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Abstract: Hybridization between native and alien congeners may pose a serious threat to biodiversity and negatively affect native flora. Here we study *Solidago ×niederederi*, which originated and became established in Europe as a result of a cross between the alien *S. canadensis* and native *S. virgaurea*. The recent increase in the number of records of *S. ×niederederi* in Europe has highlighted the need to monitor its occurrence, spread and behaviour. In the present study, we tested the effectiveness of flow cytometry for detecting hybrid plants of *S. ×niederederi*. Sequences of the ITS region of nrDNA and the *rpS15-ycf1* spacer of cpDNA were used to confirm the hybrid origin of analysed plants and to identify the maternal species. Our study included 60 single-species populations of *S. canadensis*, *S. gigantea* and *S. virgaurea,* and 16 mixed populations with the presence of hybrid *S. ×niederederi* sampled from six countries in central Europe and adjacent areas. All individuals of *S. canadensis*, *S. ×niederederi* and *S. virgaurea* investigated were diploid (2n~2x~18) but differed in their relative DNA content values. The DNA content of *S. ×niederederi* was intermediate between *S. canadensis* and *S. virgaurea* with no overlaps, with the differences between the species being statistically significant. Therefore, we conclude that flow cytometry is a reliable and efficient method for detailed screening for hybrids within mixed *Solidago* populations and for identifying non-flowering or morphologically ambiguous *Solidago* plants. Since both parental species varied only negligibly in their DNA content, it may also be applicable across a broader geographic scale. Genetic, flow cytometric and distributional data suggest that the hybrids are to a large extent early generation (likely F1) hybrids as very few cases of supposed introgressants were also inferred. The results from chloroplast *rpS15-ycf1* spacer showed that hybridization has occurred in both directions.

Keywords: homoploid hybridization, DAPI flow cytometry, nrDNA ITS, plastid DNA sequences, relative DNA content

Received 1 Jun 2021, Revised 15 Oct 2021, Accepted 20 Oct 2021, Published: 24 Mar 2022

Introduction

Hybridization, resulting from mating between individuals of different species or genetically divergent individuals within a species, is one of the most important processes in plant evolution and speciation, influencing the evolutionary course of at least 25% of plant species (Mallet 2005, Whitney et al. 2010, Abbott et al. 2013). Besides natural phenomena bringing previously isolated species/lineages into contact (e.g. range shifts due to climate oscillations; Araújo & Luoto 2007), human-induced secondary contacts have become the most influential in this regard since the Age of Discovery (15th century). Related yet different species can meet when crops (Ellstrand et al. 2013) and ornamental (e.g. Lehan et al. 2013, Pergl et al. 2016) plants are purposefully introduced; however, unintentional introductions accompanying human movement between countries are also very common (e.g. Lehan et al. 2013).

Hybridization of native and non-native congeners deserves special consideration; evidence of such cases has increased exponentially over the last few decades (Mooney & Cleland 2001, Largiadèr 2008). Hybridization can have diverse outcomes including serious conservation concerns (Rhymer & Simberloff 1996, Vilà et al. 2000). (i) One of the outcomes may be the establishment of a new (hybridogenous) species that can either remain at its place of origin (Abbott 1992), or, become invasive and adversely affect whole ecosystems and communities [e.g. *Sporobolus anglicus* (C. E. Hubb.) P. M. Peterson & Saarela – CABI 2021a], and, in extreme cases, spread so successfully that it completely displaces its parental species (Hegde et al. 2006). (ii) Gene flow between parental taxa causes ecological and genetic changes in both the introduced and native species (Strauss et al. 2006). Introgression of genes from a native species provides exotic species with preadapted genes for new environments that may enhance its invasiveness (Ellstrand & Schierenbeck 2000). On the other hand, gene flow in the opposite direction may cause erosion of the genetic pool, loss of genetic variability and thus negatively affect locally adapted populations of a native species (especially pronounced in small populations/rare species but even large native populations over the long term; Vilà et al. 2000, Wolf et al. 2001). This may in some cases lead to extinction via hybridization (Todesco et al. 2016, Vallejo-Marín & Hiscock 2016). (iii) In many cases, hybridization may be simply rare with little (long-term) effect on either parental taxon (e.g. Vallejo-Marín & Hiscock 2016, Dormontt et al. 2017).

Considering the above-mentioned threats, preventing the spread of new hybrid lineages requires quick recognition and consequently a rapid management response. Hybrids are often identified in the field based on their intermediate morphology and confirmed by genetic markers or a combination of both (Abbott et al. 2010, Saltonstall et al. 2014, Zaya et al. 2015, Fukatsu et al. 2019). Hybrid origin is also frequently accompanied by decreased fertility or even complete sterility as a result of reproductive isolating barriers (Baack et al. 2015). As hybridization and establishment of a hybrid lineage is often connected to genome duplication (allopolyploidization, e.g. Ainouche et al. 2004, Mandáková et al. 2019), or hybrids arise between congeners of different ploidies (heteroploid hybridization, e.g. Zozomová-Lihová et al. 2014, Musiał et al. 2020), karyological analysis (chromosome counts and/or nuclear DNA content) is another way of identifing hybrids. Although recognition of hybrids at the homoploid level is trickier and requires high quality of analyses (Loureiro et al. 2010), the effectivity of flow

cytometry (FCM) for detection of homoploid hybrids in natural populations has been repeatedly proven for systems in which genome sizes of the two progenitors differ significantly (e.g. by at least 7%; see Loureiro et al. 2010, Hanušová et al. 2014, Macková et al. 2017, 2018, Agudo et al. 2019). However, the expected intermediate genome size in early-generation hybrids can be disrupted by several evolutionary processes such as genome rearrangements of stabilized hybrids, natural selection of hybrids, or introgression resulting in a continuous variation in genome size linking the distinct values of parental taxa, which often complicate the detection of homoploid hybrids by flow cytometry (cf. Bureš et al. 2004, Loureiro et al. 2010, Hanušová et al. 2014, Pellicer et al. 2021).

Currently, two alien species of the genus *Solidago* L., *S. canadensis* L. and *S. gigantea* Aiton, both of North American origin, are naturalized and invasive in almost all of Europe (Kabuce & Priede 2010, Kowarik 2010, CABI 2021b, c). Here, they share habitats with the European native *S. virgaurea* L., which has led to the origin of two spontaneous hybrids: *S. ×niederederi* Khek and *S. ×snarskisii* Gudžinskas & Žalneravičius. The latter, *Solidago ×snarskisii* (2n = 3x = 27), the result of the heteroploid crossing between diploid *S. virgaurea* (2n = 2x = 18) and tetraploid *S. gigantea* (2n = 4x = 36), the fertility of which is low and is currently only known from several localities in northern and eastern Europe (Gudžinskas & Žalneravičius 2016, Pliszko 2018, Musiał et al. 2020, Vinogradova & Galkina 2020). In contrast, the number of records of *S. ×niederederi* (2n = 2x = 18), which originated from the homoploid hybridization of diploid *S. canadensis* and *S. virgaurea* (both 2n = 2x = 18), has rapidly increased, mainly over the last four decades (Musiał et al. 2020, Skokanová et al. 2020b). *Solidago ×niederederi* was noticed for the first time at the end of the 19th century (Skokanová et al. 2020a) and was recently reported from more than 400 localities in 17 European countries (Skokanová et al. 2020b). *Solidago ×niederederi* seemingly originated through multiple events of hybridization mainly at localities where both parental species grow in close proximity with one another (Pliszko & Zalewska-Gałosz 2016, Galkina & Vinogradova 2019). Further spreading of these hybrids and formation of their own coherent populations cannot be excluded in the near future because plants of *S. ×niederederi* produce viable pollen (< 70%; Migdałek et al. 2014, Karpavičienė & Radušienė 2016). A low percentage of well-developed fruits (6%; Migdałek et al. 2014, Pliszko & Kostrakiewicz-Gierałt 2017) is partially balanced by high seed germinability (91%; Pliszko & Kostrakiewicz-Gierałt 2017). *Solidago ×niederederi* has the potential to successfully combine the astonishing phenotypic plasticity of *S. virgaurea* (Turesson 1925, Takahashi & Matsuki 2016, Hirano et al. 2017, Nardi et al. 2018) with the high invasiveness of *S. canadensis* (CABI 2021b). Assuming that the hybrids and introgressed progeny may benefit from a broader range of environmental conditions than their parental taxa (cf. Bleeker et al. 2007, Currat et al. 2008, Abbott et al. 2013), *S. ×niederederi* may soon pose a serious threat to biodiversity at the species and habitat level. Therefore, monitoring the occurrence and spread of *S. ×niederederi* is an important precaution if its potential negative effect on native European ecosystems is to be mitigated.

Starting from previous reports on genome size differences between *S. canadensis* and *S. virgaurea* (Szymura et al. 2015, Fernandez et al. 2018 and references therein) and on determination of the hybrid origin of *S. ×niederederi* using the internal transcribed spacer region (ITS) of nuclear ribosomal DNA (nrDNA) or non-coding regions of chloroplast DNA (cpDNA; Pliszko & Zalewska-Gałosz 2016, Galkina & Vinogradova

2019), the main goals of the present study are: (i) test whether the hybrid plants of *S.* ×*niederederi* have intermediate values of the relative DNA content of their parental species, and consequently, if FCM could be unequivocally used for hybrid detection; (ii) test if the *Solidago* species co-occuring with *S.* ×*niederederi, i.e. S. canadensis, S. gigantea* and *S. virgaurea,* differ significantly in their relative DNA contents without any overlaps even when more extensive sampling over large areas is taken into account; (iii) verify the hybrid origin of the *S.* ×*niederederi* plants using the ITS region of nrDNA and, in addition, determine whether there is any evidence of backcrossing or later-generation hybrids in the ITS variation patterns; (iv) identify non-coding regions in cpDNA that are highly polymorphic and could differentiate between the parental species and hence reveal the direction of hybridization. To study the differentiation of genome size in the *Solidag*o taxa studied, we employed DAPI flow cytometry, which is accurate and particularly useful for detecting small differences in genome size (Marhold et al. 2010, Suda et al. 2010). Chromosome counting was used for *S.* ×*niederederi* and its parental species to verify the flow cytometry data.

Material and methods

Studied taxa

Solidago canadensis occurs naturally in the north-eastern part of the United States and southern regions of Canada. Its secondary occurrence is known from Asia, Australia, Europe, and New Zealand (Semple 2021). This species was introduced to England as early as 1645 (Kowarik 2010), afterwards, it spread as it is an attractive ornamental plant to botanical as well as common gardens and nurseries throughout Europe. In Europe, it was recorded in the wild for the first time in 1850, and an exponential increase in the number of its sites started in 1870–1900 (Weber 1998). Today, this species is naturalized and invasive from northern Italy to southern Scandinavia (Kabuce & Priede 2010). *Solidago canadensis* is exclusively diploid (2n = 2x = 18). In its native area, two varieties, *S. canadensis* var. *canadensis* and *S. canadensis* var. *hargeri* Fernald, differing mainly in the type of stem indumentum, are recognized (Melville & Morton 1982, Semple & Cook 2006). The infraspecific classification of European invasive populations of *S. canadensis* is still uncertain (Verloove et al. 2017).

Solidago virgaurea is a highly polymorphic complex widespread in temperate and cold climates in Europe and Asia and has a patchy distribution also in the Mediterranean region of southern Europe, the north-western part of Africa and Asia Minor. This complex comprises 14–24 exclusively diploid (2n = 2x = 18) subspecies and microspecies. In Europe, the following subspecies are recognized within *S. virgaurea*: *S. virgaurea* subsp. *virgaurea* widely distributed in Europe; *S. virgaurea* subsp. *minuta* (L.) Arcang. with a patchy distribution in European mountains; *S. virgaurea* subsp. *pineticola* Sennikov from the Baltic region; *S. virgaurea* subsp. *lapponica* (With.) Tzvelev from northern Europe; *S. virgaurea* subsp. *litoralis* (Savi) Briquet et Cavillier; *S. virgaurea* subsp. *macrorrhiza* (Lange) Nyman and *S. virgaurea* subsp. *rupicola* (Rouy) Lambinon from sandy coasts of France and Italy (Yuzepchuk 1959, Slavík 2004, Greuter 2006–2020, Nardi et al. 2018, Semple 2021, Tela Botanica 2021).

Solidago ×niederederi is a nothotaxon resulting from the homoploid crossing (at the diploid ploidy level) of alien *S. canadensis* and native *S. virgaurea* (Musiał et al. 2020). It is characterized by having a morphology that is intermediate between that of its parental species (Fig. 1B), especially in respect to the height of the plant and size of the inflorescence and capitulum (Karpavičienė & Radušienė 2016). In addition, plants of *S. ×niederederi* are conspicuous in the frequent presence of vegetative shoots with densely crowded leaves (in the form of a pseudorosette) at the apex (Gudžinskas & Žalneravičius 2016). The hybrid occurs mainly at sites inhabited by both parental species and only sporadically at sites inhabited by one parental species. The known occurrences of *S. ×niederederi* are restricted to a part of Europe between 45.8 and 63.8°N (Skokanová et al. 2020b). Here *S. niederederi* is classified as an established alien (Musiał et al. 2020).

The native range of *Solidago gigantea* is in southern Canada and the central and eastern part of the USA. Nowadays this species is naturalized and invasive in Europe, East Asia, the Azores and New Zealand. In Europe, *S. gigantea* was introduced in 1758 and afterwards distributed as an ornamental to gardens and nurseries (Weber & Jacobs 2005). The first observations of *S. gigantea* in the wild in Europe date back to 1850 (Weber 1998). Since then the number of its localities have exponentially increased and it has spread across almost the whole of Europe (except the north). In its native range, two varieties, var. *gigantea* and var. *shinnersii* Beaudry, both including diploid, tetraploid and hexaploid ploidy levels (only rarely triploid and pentaploid) are reported (Semple 2021). Its invasive populations in Europe belong to var. *gigantea* and are predominantly tetraploid (Schlaepfer et al. 2008a, Semple 2021). *Solidago gigantea* often co-occur with *S. canadensis*, *S. ×niederederi* and/or *S. virgaurea*.

The species studied are long-lived perennials, 0.3–2.5 m tall with 1–40 flowering shoots, capitula are relatively small but numerous (20–2000) (Slavík 2004, Skokanová 2022). All species are obligate outbreeders and are pollinated by a wide range of insects. Plants produce up to 27,700 fruits of small size. Fruits have a pappus and are easily dispersed by wind over long distances. Non-native *S. canadensis* and *S. gigantea* also reproduce asexually via underground rhizomes (Werner 1980, Huang et al. 2007, Moravcová et al. 2010, CABI 2021b, c).

Sampling

The sampling was primarily focused on mixed populations of *S. canadensis* and *S. virgaurea* with the presence of hybrid *S. ×niederederi*. Altogether, material from 248 plants originating from 16 mixed populations from Poland, Slovakia, Hungary, and Romania were collected (Fig. 1A, Table 1). The occurrence of *S. ×niederederi* in populations BST, JEL, NSB, BTL, VPA and RZC (Table 1) is reported here for the first time; the remaining localities of *S. ×niederederi* were reported previously (Pliszko et al. 2018, Skokanová et al. 2020b). In cases when *S. gigantea* was present at a site with a mixed population, we also collected plant material of this taxon.

Additional material from 246 plants originating from single-species populations of *S. canadensis*, *S. gigantea* and *S. virgaurea* (20 populations per taxon) from a broader area (Czechia, Slovakia, Hungary, Croatia and Romania; Fig. 1A, Table 1) was collected and analysed to clarify whether the origin of plant material could affect the results due to geographically conditioned variations in genome size.

Table 1. Population (Pop.) code, number (N) of plants included in relative DNA content analyses (FCM), chromosome counts (chrom.), ITS and *rpS15-ycf1* analyses, and locality details, including geographical coordinates, altitude, date and collectors of *Solidago* taxa. Relative DNA content, chromosome numbers and cpDNA haplotypes are given for particular taxa within mixed populations as well as from single species populations. *Taxon codes: C – *S. canadensis*; G – *S. gigantea*; N – *S.* ×*niederederi*, flowering individuals; Nv – *S.* ×*niederederi*, non-flowering individuals in the vegetative stage; V – *S. virgaurea*; I_C, I_N – supposed introgressants of *S. canadensis* and *S.* ×*niederederi*. **Collector abbreviations: BŠ – B. Šingliarová, JD – J. Dušek, JK – J. Kochjarová, JO – J. Olšavská, KP – K. Pulišová, KS – K. Skokanová, PM – P. Mereďa Jr., SM – S. Mikita, SŠ – S. Španiel, VK – V. Kolarčik.

Pop. code	Taxon code*	N FCM (RSS mean±SD)	N chrom.	N ITS	N *rpS15-ycf1* (cp DNA haplotypes)	Locality description, date, collector(s) **
Solidago – mixed populations						
ABU	C	5 (0.818±0.005)	1 (2n = 18)	3	3 (H9)	Hungary; Abaújvár, W of the village; 48.506699°N, 21.32746°E; 212 m; 29.8.2019; KS
	N	1 (0.858)		1	1 (H1)	
	V	4 (0.911±0.01)		2	2 (H1)	
	G	4 (1.464±0.000)				
ARP	C	4 (0.826±0.006)		2		Romania; Arpaşu de Sus, S of the village; 45.71156°N, 24.61177°E; 553 m; 15.9.2019; KS
	N	1 (0.86)		1	1 (H9)	
	V	5 (0.918±0.01)		2	2 (H1)	
	G	1 (1.473)		1	1 (H10)	
BST	C	3 (0.810±0.002)		1	1 (H6)	Slovakia; Banská Štiavnica, W part of the town, Pod Trojickým vrchom Street; 48.4471433°N, 18.898096°E; 639 m; 14.8.2020; KS, SŠ
	I_C	1 (0.819)		1	1 (H9)	
	N	1 (0.856)		1	1 (H1)	
	V	4 (0.910±0.004)		2	1 (H1)	
BTL	C	4 (0.814±0.004)		2	2 (H7, H9)	Slovakia; Betliar, SE of the village; 48.69605°N, 20.51190°E; 323 m; 19.8.2020; KS, SŠ
	N	1 (0.863)		1	1 (H1)	
	V	4 (0.911±0.004)		2	2 (H1)	
CZA	C	4 (0.807±0.009)		3	4 (H9)	Poland; Czajowice, S of the village; 50.18400°N, 19.80865°E; 441 m; 7.9.2019; KS, PM, SŠ, BŠ
	I_C	1 (0.799)		1	1 (H8)	
	N	3 (0.866±0.006)		3	3 (H3, H6)	
	V	4 (0.915±0.004)		2	2 (H1)	
	G	1 (1.466)		1	1 (H9)	
HAN	C	7 (0.820±0.003)		2	2 (H9)	Slovakia; Handlová, W of the town; 48.71894°N, 18.7295°E; 592 m; 18.8.2018; KS
	N	2 (0.856±0.003)		2	2 (H1)	
	V	6 (0.912±0.005)		2		
JAN	C	4 (0.819±0.003)		4	4 (H6, H9)	Poland; Januszowice, W of the village; 50.25505°N, 20.04258°E; 312 m; 7.9.2019; KS, PM, SŠ, BŠ
	N/Nv	9/1 (0.863±0.007)		9/1	9/1 (H1, H9)	
	V	4 (0.913±0.005)		2	2 (H1)	
	G	4 (1.453±0.006)		3	1 (H10)	
JAW	C	6 (0.811±0.011)		3	2 (H7, H9)	Poland; Jachowka, W of the village; 49.75383°N, 19.67680°E; 569 m; 6.9.2019; KS, PM, SŠ, BŠ
	N	1 (0.857±0.00)		1	1 (H1)	
	V	4 (0.917±0.005)		2	2 (H3, H4)	
	G	4 (1.455±0.013)		2		

Pop. code	Taxon code*	N FCM (RSS mean±SD)	N chrom.	N ITS	N *rpS15-ycf1* (cp DNA haplotypes)	Locality description, date, collector(s) **
JEL	C	4 (0.811±0.005)		2	2 (H9)	Slovakia; Jelšava, N of the town; 48.64153°N, 20.24199°E; 294 m; 17.8.2020; KS, SŠ
	N	1 (0.865)		1	1 (H1)	
	I_N	1 (0.841)		1	1 (H1)	
	V	4 (0.905±0.003)		1	2 (H1)	
MYD	C	7 (0.820±0.01)		2	2 (H5, H6)	Poland; Mydlniki, Krakow, N of the city district; 50.089781°N, 19.843067°E; 216 m;
	I_C	1 (0.824)		1	1 (H5)	7.9.2019; KS, PM, SŠ, BŠ
	N/Nv	4/6 (0.865±0.007)		4/6	4/6 (H1)	
	V	4 (0.915±0.006)		2	2 (H1)	
	G	4 (1.449±0.012)		2		
NEZ	C	19 (0.815±0.007)	3 (2n = 18)	6	16 (H1, H6, H7, H9)	Slovakia; Nezbudská Lúčka, loco dicto Za vodou and Hradský potok Creek valley;
	N/Nv	3/1 (0.873±0.003)	1 (2n = 18)	2/1	2/1 (H1, H9)	49.172162°N, 18.879831°E; 364–400 m; 8.9.2019; KS, PM, SŠ
	V	20 (0.919±0.009)	4 (2n = 18)	2	6 (H9)	
NSB	C	4 (0.812±0.009)		2	2 (H6)	Slovakia; Nižnoslánska Baňa, S part of the village; 48.73687°N, 20.41457°E; 389 m;
	N	4 (0.858±0.007)		4	4 (H1)	19.8.2020; KS, SŠ
	V	4 (0.915±0.004)		2	1 (H1)	
PLC	C	5 (0.812±0.007)		3	1 (H9)	Poland; Palcza, N of the village, Dział Polecki settlement; 49.82612°N, 19.73678°E; 472 m;
	N	1 (0.858)		1	1 (H1)	6.9.2019; KS, PM, SŠ, BŠ
	V	4 (0.916±0.004)		2	1 (H3)	
	G	4 (1.474±0.016)		1		
PWO	C	4 (0.818±0.013)		4	4 (H1, H3)	Poland; Podlesna Wola, W of village; 50.41077°N, 20.01547°E; 339 m; 7.9.2019; KS, PM,
	N	3 (0.870±0.008)		3	3 (H1)	SŠ, BŠ
	V	4 (0.912±0.002)		2	2 (H1)	
RZC	C	4 (0.812±0.004)				Poland; Rozcięta, S of the village; 49.72444°N, 19.32022°E; 468 m; 25.9.2020; KS, SŠ
	N	3 (0.865±0.006)				
	V	4 (0.918±0.006)				
VPA	C	4 (0.816±0.008)		2	2 (H9)	Slovakia; Hrhov, S of the village, Veľký Paklán hill; 48.58418°N, 20.73478°E; 196 m;
	N	4 (0.867±0.006)		4	4 (H9)	18.8.2020; KS, SŠ
	V	4 (0.912±0.007)		1	2 (H1, H2)	
	G	4 (1.468±0.015)		2	2 (H7, H9)	
Solidago canadensis – single species populations						
ABO	C	4 (0.826±0.005)				Hungary; Abony, N of the town; 47.21983°N, 20.01995°E; 89 m; 26.8.2018; KS
BAB	C	10 (0.81±0.006)		2	2 (H6, H7)	Slovakia; Banská Bystrica, between SC Europa and Belveder; 48.731553°N, 19.134420°E; 370 m; 17.8.2017; KS, JD
BRE	C	4 (0.820±0.005)		2	2 (H9)	Slovakia; Breznička, E part of the village; 48.42442°N, 19.74618°E; 219 m; 23.8.2019; KS, JO
COR	C	4 (0.818±0.004)				Romania; Cornu de Jos; 45.14794°N, 25.69652°E; 419 m; 16.9.2019; KS, SM
DRG	C	4 (0.818±0.004)				Slovakia; Dargovský priesmyk pass; 48.74341°N, 21.52951°E; 433 m; 1.9.2019; KS
DRI	C	4 (0.823±0.003)				Slovakia; Drietoma, N of the village towards state border; 48.94525°N, 17.92261°E; 292 m; 18.8.2018; KS
HEV	C	4 (0.815±0.005)				Hungary; Heves, SE part of the town; 47.59120°N, 20.29793°E; 88 m; 25.8.2018; KS
KRC	C	4 (0.815±0.005)				Slovakia; Kráľovský Chlmec, NE part of the town; 48.42979°N, 21.98214°E; 101 m; 30.8.2019; KS

Pop. code	Taxon code*	N FCM (RSS mean±SD)	N chrom.	N ITS	N *rpS15-ycf1* (cp DNA haplotypes)	Locality description, date, collector(s) **
KRL	C	4 (0.814±0.006)		3	2 (H6)	Slovakia; Kráľova Lehota, near railway station; 49.02703°N, 19.78339°E; 657 m; 14.8.2019; KS, JK, KP
	I_C	1 (0.819)				
LAP	C	4 (0.809±0.009)				Romania; between villages of Lăpuş and Rogoz; 47.4722°N, 23.97188°E; 364 m; 21.9.2019; KS, SM
LPN	C	4 (0.815±0.013)		2	2 (H6)	Slovakia; between villages of Lipany and Kamenica; 49.1775088°N, 20.9542215°E; 443 m; 17.6.2020; KS, SŠ
MOC	C	4 (0.810±0.009)				Romania; Mocod, N of the village; 47.26707°N, 24.29732°E; 329 m;21.9.2019; KS, SM
RIC	C	4 (0.811±0.01)				Czechia; Říčany, E margin of the village; 49.21356°N, 16.403°E; 324 m; 18.8.2018; KS
RVC	C	4 (0.819±0.006)		2	2 (H1, H9)	Slovakia; Revúca; 48.677913°N, 20.125706°E; 306 m; 17.5.2020; KS, SŠ
SIO	C	3 (0.813±0.002)				Hungary; between villages of Siófok and Fokihegy; 46.89348°N, 18.05007°E; 107 m; 29.8.2018; KS
SZI	C	3 (0.808±0.005)				Hungary; Szikszó, S of the village; 48.17715°N, 20.91450°E; 111 m; 1.10.2017; KS, VK
TRE	C	4 (0.813±0.002)				Czechia; Třebíč, E part of the town; 49.21661°N, 15.90361°E; 389 m; 19.8.2018; KS, SM
VAN	C	4 (0.825±0.005)				Romania; Vânători, W of the village; 46.23738°N, 24.90969°E; 387 m; 20.9.2019; KS, SM
VBY	C	4 (0.81±0.001)				Slovakia; Bytča, N of the town; 49.24181°N, 18.55254°E; 322 m; 19.8.2019; KS
ZBJ	C	4 (0.812±0.007)				Slovakia; Zboj, SW of the village; 49.021385°N, 22.472045°E; 340 m; 6.7.2019; KS
Solidago virgaurea – single species populations						
ALA	V	4 (0.924±0.005)				Croatia; Velebit Mts, Alaginac pass; 44.546312°N, 15.168191°E; 1150 m; 18.6.2019; KS, JO
BEC	V	4 (0.900±0.001)		2	2 (H1)	Slovakia; Beckov, S of the village, Beckovské Skalice nature reserve; 48.77447°N, 17.89199°E; 190 m; 28.8.2020; KS, SŠ
BIA	V	4 (0.912±0.005)				Poland; Nowa Biała, parking lot near Przelom Bialky; 49.42855°N, 20.12408°E; 625 m; 14.8.2019; KS, JK, KP
CRM	V	4 (0.926±0.015)				Romania; Bucegi Mts, close to Cabana Caraiman; 45.410478°N, 25.487389°E; 2130 m; 17.9.2019; KS, SM
DSP	V	4 (0.924±0.005)				Slovakia; Belianske Tatry Mts, Dolina siedmych prameňov valley; 49.22697°N, 20.27944°E; 1520 m; 15.8.2019; KS, JK, KP
HUB	V	4 (0.909±0.004)				Slovakia; Hubina, SE of the village, valley above Striebornica dam; 48.60794°N, 17.92528°E; 293 m; 21.8.2018; KS, SM
JAL	V	4 (0.912±0.008)				Slovakia; Banská Bystrica, Jakub quarter, site Jakubské lúky; 48.765893°N, 19.14336°E; 433 m; 7.6.2019; KS
JVR	V	4 (0.917±0.001)				Slovakia; Uhrovec, E of the village, close to Jankov vŕšok hill; 48.739925°N, 18.390209°E; 640 m; 30.5.2020; KS
KJS	V	4 (0.913±0.004)		3	1 (H1)	Slovakia; Kojšov, E of the village, below Zemničky saddle; 48.824287°N, 21.011266°E; 709 m; 12.5.2020; KS
KKO	V	4 (0.929±0.003)				Slovakia; Západné Tatry Mts, S slopes of Mt. Kondrátova kopa; 49.23481°N, 19.93175°E; 1957 m; 16.8.2019; KS, JK, KP
LSC	V	4 (0.914±0.005)				Slovakia; Veľká Fatra Mts, below the top of Mt. Lysec; 48.996177°N, 19.065547°E; 1325 m; 22.6.2016; KS
MRK	V	4 (0.918±0.001)		1	1 (H1)	Slovakia; Veľký Kolačín, SE of the village, SE slope of Markovnica hill; 48.925081°N, 18.19405°E; 530 m; 12.5.2020; KS

Pop. code	Taxon code*	N FCM (RSS mean±SD)	N chrom.	N ITS	N *rpS15-ycf1* (cp DNA haplotypes)	Locality description, date, collector(s) **
MRN	V	4 (0.926±0.003)				Slovakia; Muráň, NE of the village, site Šiance; 48.767778°N, 20.079278°E; 683 m; 12.7.2019; KS, JK
PLE	V	4 (0.906±0.005)				Croatia; Jastrebarsko, Plešivica settlement; 45.725393°N, 15.656632°E; 325 m; 12.8.2018; KS
RAB	V	4 (0.921±0.003)				Slovakia; Poloniny Mts, Mt. Rabia skala; 49.100927°N, 22.463959°E; 1089 m; 5.7.2019; KS
SAN	V	3 (0.905±0.006)				Slovakia; Devínska Nová Ves, S of the village, Sandberg nature reserve; 48.201563°N, 16.974144°E; 197 m; 9.5.2019; KS
SHO	V	4 (0.920±0.004)		2	1 (H1)	Slovakia; Liptovská Teplička, spring of the river Hornád; 48.98089°N, 20.10653°E; 914 m; 31.8.2020; KS, SŠ
TAT	V	4 (0.909±0.003)				Slovakia; Štiavnické vrchy Mts, Tatárska lúka; 48.405194°N, 18.870861°E; 890 m; 1.5.2019; KS
TFG	V	3 (0.925±0.01)				Romania; Făgăraş Mts, Transfăgăraşan; 45.65128°N, 24.60513°E; 1151 m; 15.9.2019; KS
TRN	V	4 (0.917±0.005)				Slovakia; Krahule, NW of the village, Trnovník hill; 48.7360203°N, 18.922294°E; 964 m; 30.5.2020; KS
Solidago gigantea – single species populations						
ALB	G	4 (1.474±0.006)				Hungary; Albertirsa, near sports stadium; 47.25215°N, 19.61763°E; 120 m; 26.8.2018; KS
BAL	G	4 (1.490±0.005)				Hungary; Baltonbevény, SE of the village; 46.67373°N, 17.34463°E; 198 m; 29.8.2018; KS
DCE	G	4 (1.461±0.015)				Croatia; Donja Čemerica, NW of the settlement; 45.33235°N, 15.921917°E; 156 m; 21.6.2019; KS, JO
HUM	G	4 (1.464±0.008)				Slovakia; Humenné, W of the town; 48.929°N, 21.86554°E; 181 m; 4.7.2019; KS
CHA	G	4 (1.459±0.015)				Slovakia; Chľaba, N of the village; 47.8364093°N, 18.8249918°E; 107 m; 18.5.2020; KS, SŠ
CHO	G	4 (1.475±0.011)				Slovakia; Chotín, near the railway station; 47.80647°N, 18.18978°E; 107 m; 21.7.2020; KS
KOZ	G	4 (1.485±0.011)				Czechia; Kožušnice, N part of the village; 49.15711°N, 17.18747°E; 274 m; 18.8.2018; KS
LOP	G	4 (1.465±0.007)				Slovakia; Lopej; 48.817572°N, 19.500472°E; 451 m; 12.7.2019; KS, JK
MAN	G	4 (1.484±0.006)				Romania; Mânerău, E of the village; 46.41198°N, 22.0071°E; 143 m; 14.9.2019; KS, SM
OCU	G	4 (1.461±0.014)				Croatia; Očura, NE of the village; 46.19688°N, 15.99614°E; 257 m; 16.6.2019; KS, JO
OSW	G	4 (1.442±0.009)				Poland; Ostrowsko, W of the village, under the bridge; 49.473086°N, 20.110869°E; 567 m; 14.8.2019; KS, JK, KP
PAS	G	4 (1.470±0.029)				Czechia; Pasohlávky, W of the village; 48.90186°N, 16.55247°E; 161 m; 19.8.2018; KS, SM
PES	G	4 (1.455±0.008)				Croatia; Plesmo, S of the village; 45.30882°N, 16.83869°E; 104 m; 22.6.2019; KS, JO
PEZ	G	4 (1.455±0.011)				Slovakia; Pezinok, NW of the town, site Rudné Bane; 48.32°N, 17.23669°E; 235 m; 20.8.2018; KS, SM
REC	G	4 (1.451±0.005)				Hungary; Recsk, S of the village; 47.91817°N, 20.10032°E; 203 m; 25.8.2018; KS
SLP	G	4 (1.484±0.019)				Croatia; N of Strednji Lipovec; 45.26524°N, 17.64291°E; 211 m; 2.7.2020; KS, SŠ
SVI	G	5 (1.492±0.013)				Slovakia; Svinná, N margin of the village; 48.79514°N, 18.15519°E; 246 m; 18.8.2018; KS
SZE	G	4 (1.448±0.008)				Hungary; Székesfehérvár, SE of the town; 47.17173°N, 18.49180°E; 115 m; 29.8.2018; KS
VAS	G	4 (1.472±0.007)				Hungary; Vaspör, NW of the village, road E86; 46.922698°N, 16.600041°E; 250 m; 4.8.2019; KS
VIN	G	4 (1.476±0.007)				Slovakia; Viničky, W margin of the village, near the railways; 48.396684°N, 21.73029°E; 98 m; 30.8.2019; KS
N total	494	8	150	146		

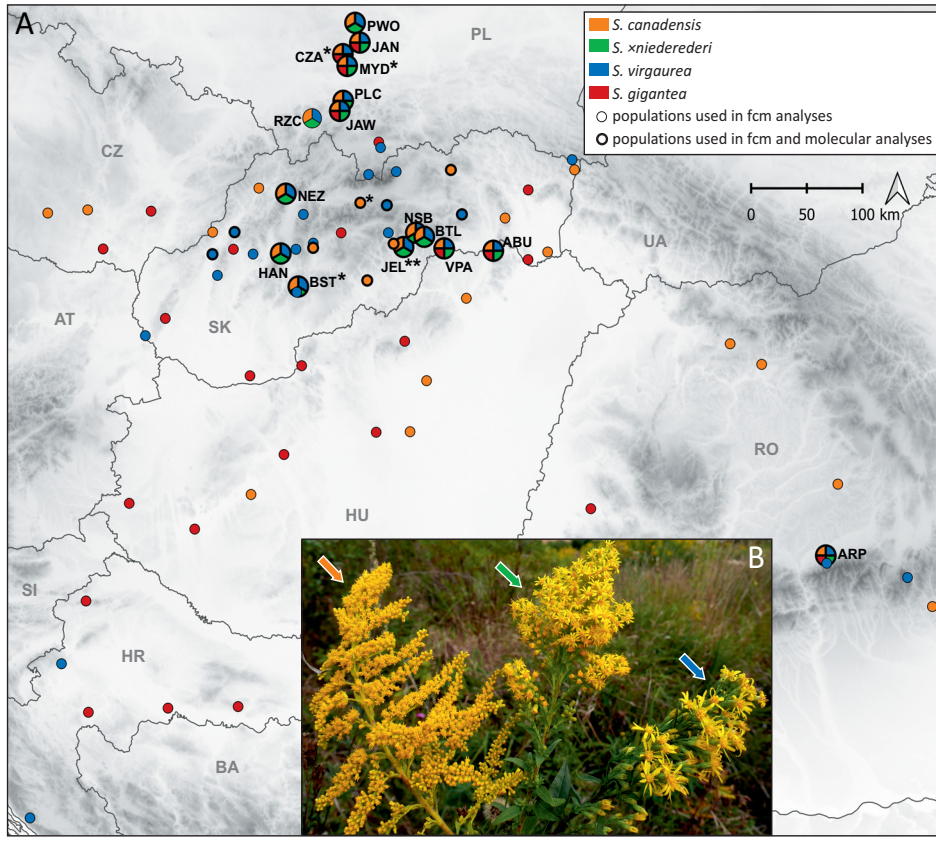

Fig. 1. (A) Map of the sites of the *Solidago* taxa analysed. Mixed populations with the hybrid *S.* ×*niedererederi* are labelled with population codes (see Table 1). Populations marked by asterisk(s) included a supposed introgressant I_C (*) or I_N (**) of *S. canadensis* and *S.* ×*niedererederi* (see Results for more details). (B) *Solidago canadensis*, *S.* ×*niedererederi* and *S. virgaurea* from the Abaújvár locality (Hungary). Photograph: K. Skokanová.

The flowering plants were determined in the field using known diagnostic morphological characters (Slavík 2004, Skokanová 2022). For identification of *S.* ×*niedererederi* we also used morphological characters identified by Gudžinskas & Žalneravičius (2016), Karpavičienė & Radušienė (2016) and Galkina & Vinogradova (2019). From four localities with mixed populations (JAN, JEL, MYD, NEZ), nine plants in the vegetative stage, suspected to be of hybrid origin due to the presence of vegetative shoots with densely crowded leaves at the apex typical of *S.* ×*niedererederi* (cf. Gudžinskas & Žalneravičius 2016), were also collected (Table 1, Supplementary Fig. S1).

Plant material was collected in the field during 2019–2020. Selected intact fresh leaves were kept in a cool place (~4–6 °C) until used in the FCM analyses; other leaves, taken from the same individuals, were dried in silica gel for molecular analyses. Some plants were transferred and cultivated in pots for chromosome counting. All collected plants were subjected to FCM analyses. A selection of plants analysed by flow cytometry was subjected to molecular analyses (sequencing of ITS and *rpS15-ycf1* regions) and chromosome counting (see below and Table 1). Voucher specimens were deposited in the

herbarium of the Institute of Botany, Plant Science and Biodiversity Centre, Slovak Academy of Sciences (SAV).

Molecular analyses (ITS of nrDNA and rpS15-ycf1 of cpDNA)

Total genomic DNA was extracted from ~3–4 mg of silica gel-dried leaf material using the DNeasy Plant Mini Kit (Qiagen, Düsseldorf, Germany).

The ITS region of nrDNA (ITS1-5.8S-ITS2) was amplified in 150 individuals a priori determined based on morphology. It included one to 10 plants per each taxon from 15 mixed populations (all except population RZC found later), comprising 44 individuals of *S. canadensis*, 47 of *S. ×niederederi*, 28 of *S. virgaurea* and 12 of *S. gigantea*, complemented by one to three plants from single-species populations of *S. canadensis* (5 populations, 11 individuals) and *S. virgaurea* (4 populations, 8 individuals) (Table 1). The PCR reaction mix contained a sample of gDNA, 0.38 U *Pfu* DNA polymerase (Promega, Madison, WI, USA), 1× *Pfu* reaction buffer with $MgSO_4$, 0.2 mM dNTPs and 0.2 mM forward and reverse primers (ITS5, ITS4; White et al. 1990), in a final volume of 13 µl. Amplification was performed under the following PCR conditions: 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, followed by 72 °C for 10 min. PCR products were purified enzymatically with a mixture of exonuclease I and FastAP thermosensitive alkaline phosphatase, according to the manufacturer's protocol (Thermo Fisher Scientific Inc., Waltham, MA, USA). The sequencing was carried out at Eurofins Genomics Company (Konstanz, Germany) using both forward and reverse primers. The resulting electropherograms were carefully inspected for the presence of double peaks (intra-individual site polymorphisms, 2ISPs, following Potts et al. 2014), and when confirmed by both forward and reverse sequences, were coded using IUPAC ambiguity codes. The sequences were edited and aligned in Geneious v. R10 (Kearse et al. 2012). The sequence alignment was analysed using NeighbourNet (SplitsTree4 v. 4.14.4; Huson & Bryant 2006) based on uncorrected P-distances and the 'average' option for treating ambiguous bases.

Previously employed *trnL-trnF* and *rpl32-trnL*^{UAG} intergenic spacers of cpDNA (e.g. Galkina & Vinogradova 2019) did not yield sufficient resolution to differentiate between *S. virgaurea* and *S. canadensis*, so neither were suitable to determine unequivocably the maternal parent of the hybrids. Therefore, we explored several other cpDNA spacers that are ranked among the most informative (Shaw et al. 2005, 2007, 2014, Prince 2015). The *rpS15-ycf1* spacer turned out to be the most polymorphic one in the initial screening tests. Finally it was amplified in 146 individuals, which were morphologically determined and included: one to 16 plants per each taxon from 15 mixed populations (all except population RZC, found later), comprising 50 individuals of *S. canadensis*, 47 of *S. ×niederederi*, 29 of *S. virgaurea* and 5 of *S. gigantea*, as well as one or two plants from single-species populations of *S. canadensis* (5 populations, 10 individuals) or *S. virgaurea* (4 populations, 5 individuals) (Table 1). The PCR reaction mix contained a sample of gDNA, 0.65 U DreamTaq DNA polymerase (Thermo Fisher Scientific), 1× DreamTaq reaction buffer with $MgCl_2$, 0.2 mM dNTPs and 0.2 mM forward and reverse primers (rpS15, ycf1; Prince 2015), in a final volume of 13 µl. PCR cycling conditions were the same as used for the ITS region. PCR products were purified enzymatically as specified above. The sequencing was carried out at Eurofins Genomics Company (Konstanz, Germany)

mostly in one direction only (using the forward primer). The sequences were aligned in Geneious v. R10, and five indels (insertion/deletion events) identified in the alignment were coded as additional binary characters, following the simple indel coding approach of Simmons & Ochoterena (2000), and appended to the nucleotide dataset. The final dataset was analysed using the statistical parsimony network implemented in TCS v.1.21 (Clement et al. 2000). This analysis identified different haplotypes in the dataset, revealed haplotype sharing between individuals and species, and determined genetic distances (number of substitutions or indel mutations) among them. Both ITS and *rpS15-ycf1* dataset-based analyses were performed with and without sequences of *S. gigantea.* All sequences were submitted to the GenBank nucleotide database (Supplementary Table S1, MZ005322-MZ0055471 for ITS, MZ020814-MZ020959 for *rpS15-ycf1*).

Chromosome counting

For karyological analyses, root tip meristems from potted plants of *S. canadensis* (two populations, four individuals), *S.* ×*niederederi* (one individual) and *S. virgaurea* (one population, four individuals) were used (Table 1). The root tips were pre-treated in a 0.002 M water solution of 8-hydroxyquinoline at 4 °C for about 16 h (overnight), fixed in a 1:3 mixture of 98% acetic acid and 96% ethanol for 1–24 h, washed in distilled water, macerated in 1N HCl at 60 °C for 5 min and then washed in distilled water. Tip squashes were made using the cellophane square technique (Murín 1960). Permanent slides were stained with a 7% solution of Giemsa Stain – Modified Solution (Fluka Analytical), in Sörensen phosphate buffer, dried and observed in a drop of immersion oil using a Leica DM 1000 microscope equipped with an HDCE-X5 camera and ScopeImage 9.0 software and the number of chromosomes counted.

Relative DNA content

Altogether 494 plants were analysed by flow cytometry using fluorochrome 4', 6-diamidino-2-phenylindole (DAPI). Based on a priori morphological identification, the following material was included in FCM analyses: one to 20 plants per each taxon from 16 mixed populations (91 individuals of *S. canadensis*, 51 of *S.* ×*niederederi*, 83 of *S. virgaurea* and 25 of *S. gigantea*) and one to 10 (mostly 4) plants from single-species populations of *S. canadensis* (20 populations, 84 individuals), *S. virgaurea* (20 populations, 78 individuals) and *S. gigantea* (20 populations, 81 individuals) (Table 1). To ensure the accuracy of the estimates of the relative DNA content, each plant was analysed separately and only fresh plant material was used. Fresh material of *Solanum pseudocapsicum* L. (2.59 pg DNA/2C; Temsch et al. 2010a) was added for internal standardization. Nuclei isolation and staining procedure followed the simplified two-step protocol (Doležel et al. 2007) with some modifications. Intact leaf tissue of the analysed plant was chopped together with an internal standard in 1 ml of ice-cold Otto I buffer (0.1M citric acid, 0.5% Tween 20). The crude nuclear suspension was filtered through 42-μm nylon mesh. For staining, 1 ml of a solution containing Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$), 2-mercaptoethanol (2 μl/ml) and DAPI (4 μg/ml) was added to the flow-through fraction. Samples were analysed after 10 min incubation at room temperature. Fluorescence of at least 5000 particles was recorded, and only histograms with symmetrical peaks with a coefficient of variance (CV) of the standard and sample G1 peaks below 3% were considered.

Flow cytometric analyses were carried out using a Cyflow ML instrument or Cyflow Space instrument (Partec, Münster, Germany) equipped with a UV-LED as an excitation source. Flow cytometric histograms were evaluated using FloMax software v. 2.7d (Partec, Münster, Germany).

The relative DNA content was calculated as the ratio of G1 peak of standard *Solanum pseudocapsicum* and G1 peak of the *Solidago* sample (the ratio standard/sample hereinafter referred to as RSS; 2C values presented unless otherwise stated). The relationship between chromosome numbers and relative DNA content of *S. canadensis*, *S. virgaurea* and *S. ×niederederi* was verified using chromosome counts (cf. Table 1).

Box-and-whisker plots and scatter plots were used to depict variation in relative DNA content of the taxa studied; t-tests and the Tukey-Kramer test (Tukey's test for unequal sample size; Tukey 1977) were used to test for differences in relative DNA content between taxa. The normal distribution was tested a priori using the Kolmogorov-Smirnov test. Analyses were carried out using STATISTICA 12 (StatSoft Inc. 2013).

Results

Molecular analyses (ITS of nrDNA and rpS15-ycf1 of cpDNA)

The overall ITS alignment was 628 bp (base pairs) long and included 46 variable sites (Supplementary Table S2). Intraindividual site polymorphisms (2ISPs), suggesting the presence of multiple ITS copy variants in the genome, were detected in all the taxa examined. Within diploid *S. canadensis* and *S. virgaurea*, originating from both pure and mixed populations, such positions were mostly rare and scattered, with up to two 2ISPs per individual. In the tetraploid *S. gigantea*, 2ISPs were more frequent, with up to six such positions per individual, suggesting higher intragenomic ITS copy variation. The ITS sequences of *S. virgaurea* and *S. canadensis* were differentiated by seven substitutions (SNPs, all located in ITS2), which can be treated as species-specific. All individuals identified based on morphology as *S. ×niederederi* had additive 2ISPs at all these seven positions, clearly supporting the presence of both *virgaurea*- and *canadensis*-specific ITS copy variants in their genomes and thus their hybrid origin. The only exception was one non-flowering individual, SOL-N-JEL2, morphologically identified as *S. ×niederederi* (Supplementary Fig. S1C), which had an ITS sequence lacking any 2ISPs and was identical to the predominant ribotype of *S. canadensis* (the individual latter denoted as "I_N"; Supplementary Tables S2, S3). Furthermore, in four individuals classified as *S. canadensis* based on morphology (SOL-C-KRL13, SOL-C-MYD1, SOL-C-BST1 and SOL-C-CZA1), additive 2ISPs were recorded at four of the seven positions diagnostic for hybrids (these four individuals hereinafter denoted as "I_C"; Supplementary Table S3). The individuals I_C and I_N might be a result of backcrossing between *S. canadensis* and *S. ×niederederi* and hereinafter are referred to as "supposed introgressants". In the NeighborNet diagram of ITS alignment (Fig. 2; *S. gigantea* omitted), individuals of *S. canadensis* and *S. virgaurea* formed two well-differentiated, species-specific clusters, whereas individuals of *S. ×niederederi* were in an intermediate position between them, in accordance with the observed additive 2ISPs patterns. The four supposed introgressants were placed in a separate position, in between *S. ×niederederi* and *S. canadensis*. When individuals of *S. gigantea* were included in the NeighborNet analysis (Supplementary Fig. S2), they appeared in a species-specific cluster, close to *S. canadensis*.

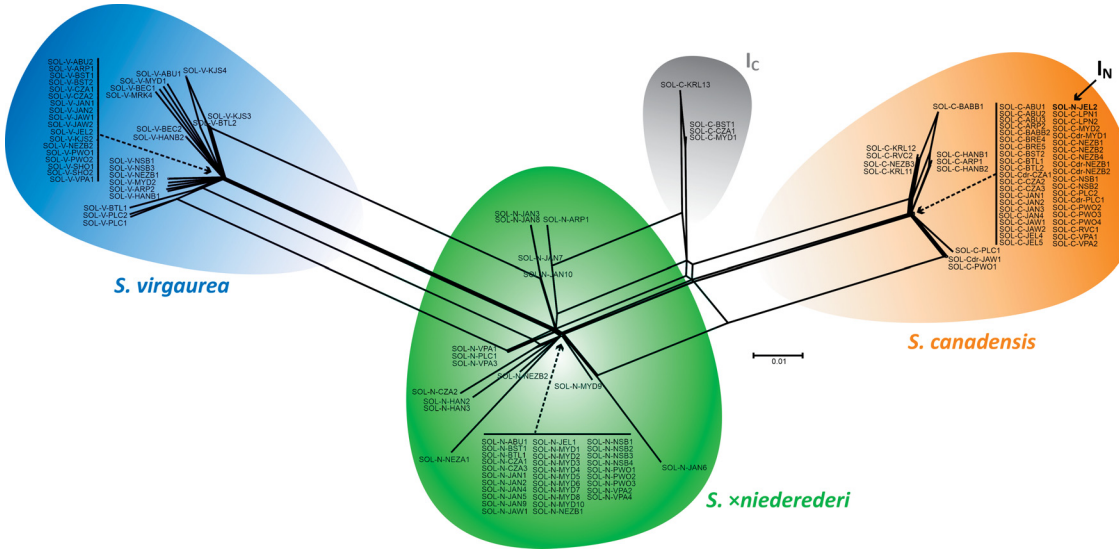

Fig. 2. NeighborNet network based on the ITS sequences of nrDNA of the *Solidago* taxa studied (*S. gigantea* not included). Terminal labels denote individual sequences (see population codes in Table 1). Three major groups of ribotypes are coloured according to the species assignment; I_C, I_N – supposed introgressants of *S. canadensis* and *S. ×niederederi.*

The overall *rpS15-ycf1* alignment was 495 bp long. It included two variable positions (substitutions) and five indels, coded as additional binary characters, which altogether resulted in ten different haplotypes (with *S. gigantea* included; nine haplotypes without *S. gigantea*; Fig. 3, Supplementary Fig. S3). Two widespread haplotypes were identified, present in 71 individuals (haplotype H1) and 44 individuals (H9). Five haplotypes were present in two to 15 individuals, and the remaining three haplotypes were individual-specific. Samples of *S. canadensis* included six different haplotypes (also including the supposed introgressants with *S. ×niederederi*); three of these appeared to be species-specific (disregarding hybrids; H5, H6, H8), two were shared with *S. gigantea* (H7, H9) and only one was shared with *S. virgaurea* (the most widespread H1). There were four different haplotypes in samples of *S. virgaurea*; three of them were species-specific (disregarding hybrids; H2, H3, H4), and one (H1) was shared with *S. canadensis* (Fig. 3, Supplementary Fig. S3). Samples of *S. ×niederederi* included four different haplotypes; two of which were shared with *S. canadensis* (H6, H9), one with *S. virgaurea* (H3) and one was the widespread one (H1) shared with both parental species. This implies that the hybridization occurred in both directions. The samples of *S. gigantea* contained either a species-specific haplotype (H10) or shared haplotypes with *S. canadensis* (H7, H9). No geographic patterns in the distribution of haplotypes were recorded, which was confirmed by a detailed screening of haplotype variation within population NEZB (Table 1). In *S. canadensis* from NEZB, as many as four different haplotypes (H1, H6, H7 and H9) were recorded. In *S. ×niederederi* from NEZB, two widespread haplotypes were recorded, H1 shared by both parental species, and H9 shared by *S. canadensis* and *S. gigantea*. These findings also indicate that a significant portion of the overall variation in haplotypes recorded here can be found within populations.

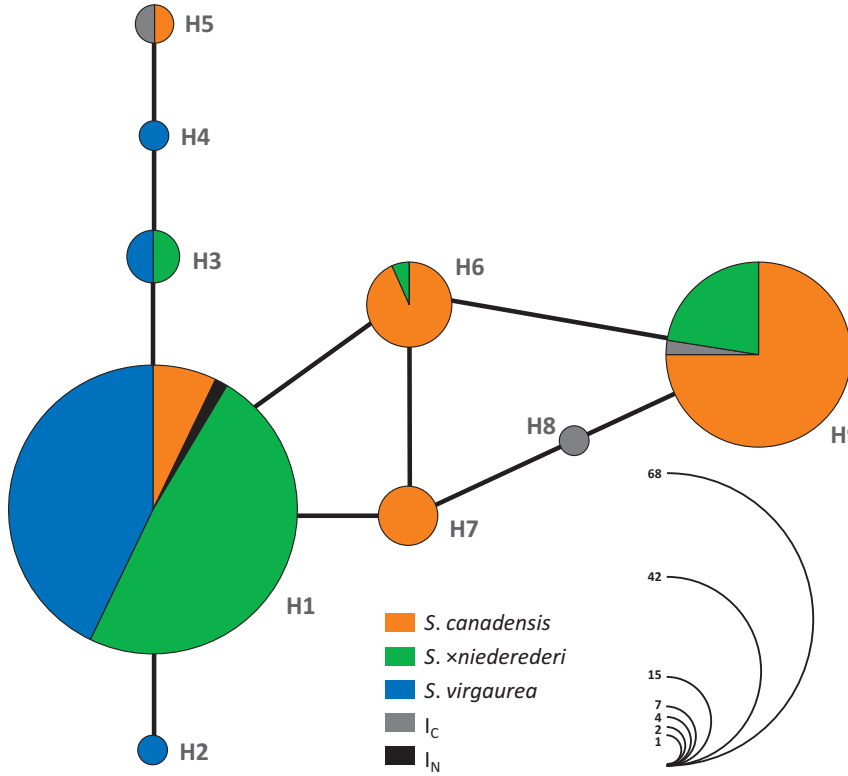

Fig. 3. Maximum parsimony network of the cpDNA haplotypes (the *rpS15-ycf1* spacer) of the *Solidago* taxa studied (*S. gigantea* not included). Circles show haplotypes (H1–H9), lines represent mutational steps; circle sizes are proportional to haplotype frequencies (see scale) and colours indicate taxon. I_C, I_N – supposed introgressants of *S. canadensis* and *S. ×niederederi.*

Chromosome counts

Chromosome numbers are newly reported for four individuals of *S. canadensis* (from populations ABU, NEZ), four individuals of *S. virgaurea* and one individual of *S. ×niederederi* (both taxa from population NEZ) (Fig. 4A–C, Table 1). Our analyses confirm chromosome counts 2n = 18 for all samples examined.

Relative DNA content

The mean CV values of the G1 peaks of the internal standard *Solanum pseudocapsicum* and *Solidago* estimates were 1.92±0.39% and 2.18±0.44%, respectively. All individuals of *S. canadensis*, *S. ×niederederi* and *S. virgaurea* examined were exclusively diploid with 2n~2x~18 (RSS 0.793–0.938). Relative DNA content estimates of *S. gigantea* corresponded to a tetraploid ploidy level 2n~4x~36 (RSS 1.432–1.507; Supplementary Fig. S4). *Solidago gigantea* significantly differed from the remaining diploid taxa in 2C values as well as in equivalent of Cx values of relative DNA content (Fig. 5A; Tukey-Kramer test, $P < 0.001$). Based on this finding the involvement of *S. gigantea* in the origin of the studied hybrid can be ruled out.

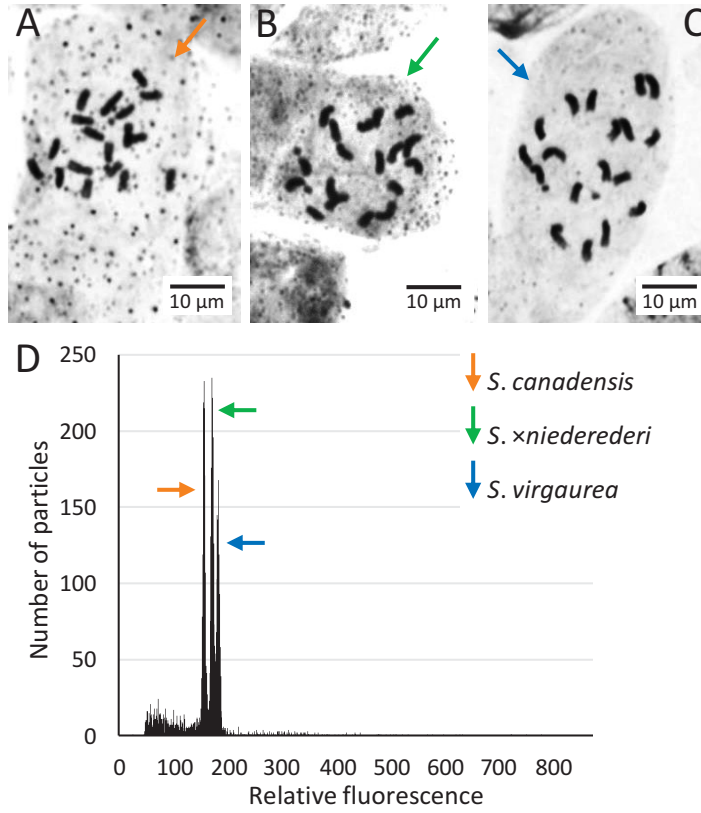

Fig. 4. Photographs of chromosome metaphase plates (2n = 18; A – SOL-C-NEZB7, B – SOL-N-NEZB1, C – SOL-V-NEZB7) and simultaneous flow cytometry analyses illustrating the differences in relative DNA content (D – SOL-C-NEZB2, SOL-N-NEZB1 and SOL-V-NEZB5) of individuals of *Solidago canadensis*, *S.* ×*niederederi* and *S. virgaurea* from the population Nezbudská Lúčka in Slovakia (NEZ).

At the diploid level (Fig. 5B, C), statistically significant differences (Tukey-Kramer, $P < 0.001$) were recorded in the relative DNA content of *S. canadensis* (RSS 0.793–0.838) and *S. virgaurea* (RSS 0.897–0.938) (Fig. 4D, E, Table 2). However, there were no statistical differences in the relative DNA content of individuals from mixed and single-species populations either of *S. canadensis* (t-test, t = –0.425, n.s.) or *S. virgaurea* (t-test, t = –1.257, n.s.; Fig. 5A, B). In general, variation in the relative DNA content within particular diploid species was low (less than 5.8%; Table 2). The relative DNA content of *S.* ×*niederederi* (RSS 0.847–0.881) was clearly intermediate between *S. canadensis* and *S. virgaurea,* as depicted by box-and-whisker plots (Fig. 5, Supplementary Fig. S4). The mean RSS value of *S.* ×*niederederi* (RSS 0.864) was negligibly lower than the expected one [RSS 0.865; (mean RSS of *S. canadensis* + mean RSS of *S. virgaurea*)/2]. Differences in relative DNA content between parental and hybrid diploid taxa were relatively low but statistically significant (Tukey-Kramer, $P < 0.001$; Fig. 4D, E). Values for *S.* ×*niederederi* were 6% higher on average than those of *S. canadensis* and 5.6% lower than those of *S. virgaurea*. A histogram of their simultaneous FCM measurements is shown in Fig. 4D. The values of the relative DNA content of *S.* ×*niederederi*

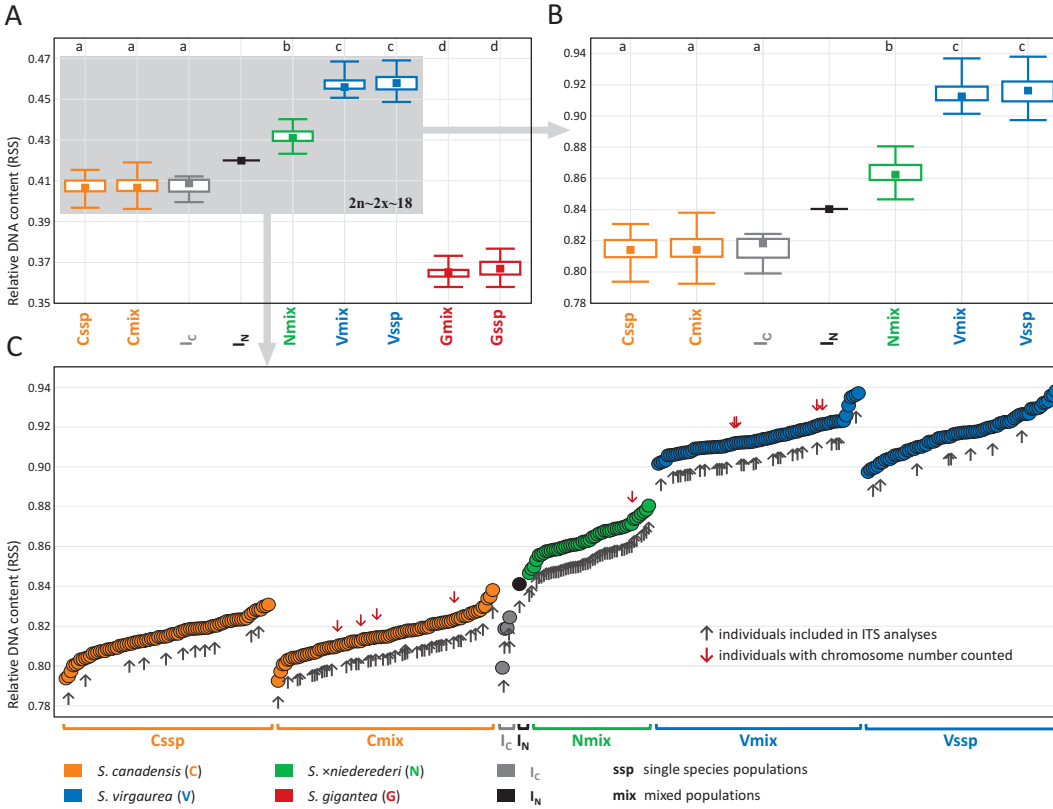

Fig. 5. Boxplots and scatter plots depicting the relative genome size (RSS) – equivalent of Cx values (A) and 2C values (B, C) measured for the *Solidago* taxa studied. Groups not significantly different at $P < 0.01$ are indicated by the same letters (Tukey-Kramer test; sample I_N was not included in the statistical tests as based on a single observation). Boxes define 25th and 75th percentiles, squares show median values, and whiskers extend from the minimum to the maximum. I_C, I_N – supposed introgressants of *S. canadensis* and *S. ×niederederi.*

Table 2. Relative DNA content of the *Solidago* taxa studied. N (ind./pop.) – number of individuals and populations of particular taxa included in relative DNA content analyses; I_C, I_N – supposed introgressants of *S. canadensis* and *S. ×niederederi*; Var. – variation [calculated as (maximum×100/minimum)-100].

Taxon (ploidy)	N (ind./pop.)	Relative DNA content (2C values)			
		Ploidy	RSS minimum–maximum	RSS mean±SD	Var.
S. canadensis	171/36	2n~2x~18	0.793–0.838	0.815±0.008	5.8%
S. ×niederederi	50/16	2n~2x~18	0.847–0.881	0.864±0.008	4.0%
S. virgaurea	161/36	2n~2x~18	0.897–0.938	0.916±0.009	4.5%
I_C	4/4	2n~2x~18	0.799–0.824	0.813±0.010	3.2%
I_N	1/1	2n~2x~18	0.841		
S. gigantea	106/28	2n~4x~36	1.432–1.507	1.467±0.018	5.3%

were clearly distinct from *S. canadensis* and *S. virgaurea* in each of the mixed populations studied (Supplementary Fig. S5).

The ITS analyses confirmed the hybrid origin (Fig. 5C, Supplementary Table S3) for almost all flowering and non-flowering individuals identified based on morphology as *S.* ×*niederederi* (ITS sequences were analysed in 46 individuals out of 50 individuals included in the FCM analyses) and with intermediate relative DNA content values between *S. canadensis* and *S. virgaurea*. In addition, the exceptional non-flowering SOL-N-JEL2 individual identified based on morphology as *S.* ×*niederederi,* but with an ITS sequence identical to *S. canadensis* (supposed introgressant $I_{N,}$ see above) had a relative DNA content intermediate between *S.* ×*niederederi* and *S. canadensis* (RSS 0.841, Fig. 5). The relative DNA content of the other four supposed introgressants I_C, classified based on morphology as *S. canadensis,* but with additive 2ISPs in ITS sequences at four of seven positions diagnostic for hybrids, was in the range of *S. canadensis* (RSS 0.799–0.824; Fig. 5, Table 2). The ITS analyses confirmed the classification of the remaining diploid individuals included in FCM analyses as *S. canadensis* (ITS sequences analysed in 51 individuals out of 171 individuals included in FCM analyses) or *S. virgaurea* (ITS sequences analysed in 36 individuals out of 161 individuals included in FCM analyses) (Fig. 5C, Supplementary Fig. S5).

Discussion

Ploidy levels and chromosome counts for the Solidago species studied

Diploid chromosome numbers/ploidy levels (2n = 2x = 18, 2n~2x~18) of *S.* ×*niederederi* detected in the present study agree with the previously published data for 32 populations from Austria, Latvia, Lithuania, and Poland (Karpavičienė & Radušienė 2016, Musiał et al. 2020). Similarly, the diploid level (2n = 2x = 18, 2n~2x~18) here detected for *S. virgaurea* is the same as ~75 previously published chromosome records for this species from Europe (Goldblatt & Johnson 1979–2021, Rice et al. 2015, Szymura et al. 2015, Nardi et al. 2018, Watanabe 2021). The only outstanding reports for *S. virgaurea* in Europe are three decaploid (2n~10x~90) plants reported from a serpentine site in Bosnia and Herzegovina (Pustahija et al. 2013). For *S. canadensis*, 23 chromosome/ploidy level records are available from Europe (cf. Szymura et al. 2015, Watanabe 2021), all of them reporting a diploid ploidy level (2n = 2x = 18) for this species and thus they are in accord with our results. The species is today considered to be exclusively diploid (Semple 2021); a tetraploid chromosome count (2n = 36) was also previously reported for *S. canadensis* (Taylor & Mulligan 1968, Semple & Chmielewski 1987). *Solidago gigantea* comprises in North America more or less spatially segregated diploid, tetraploid and hexaploid populations together with the rare occurrence of triploids and pentaploids (e.g. Semple et al. 1993, 2001, Schlaepfer et al. 2008a, Morton et al. 2019, Semple 2021). But, only tetraploid plants of *S. gigantea* are confirmed by cytogeographic studies within its invasive range in Europe, Russia and Japan (Schlaepfer et al. 2008a) and in south-western Poland (Szymura et al. 2015). Some previous records of diploid and hexaploid plants of *S. gigantea* in Europe (Maurer 1987 as cited in Jurenitsch et al. 1988, Jakobs 2004) are ruled out based on re-examinations of original material or material from the provided localities. These records were attributed to misidentifications, or technical failings (Schlaepfer et al. 2008a). Later, Hull-Sanders et al. (2009) report one diploid and six diploid-tetraploid populations of *S. gigantea* from France, Germany and Switzerland, however, possible

contamination of seed material or confusion with *S. canadensis* was not considered in their study. In the present study, we recorded only the tetraploid ploidy level for 28 populations (81 plants) of *S. gigantea* from Slovakia, Poland, Hungary and Croatia, an area poorly represented in previous studies (cf. Schlaepfer et al. 2008a, Szymura et al. 2015 and references therein).

Aneuploidy in the strict sense (the presence of supernumerary A chromosomes) is currently unknown in the *Solidago* taxa studied, but supernumerary B chromosomes have been reported several times for *S. canadensis* (Kapoor 1978, Małecka 1989, Albers & Bennert 1998), *S. gigantea* (Semple et al. 1984) and *S. virgaurea* (Lövkvist & Hultgård 1999). Although B chromosomes were not detected in the present study, either by chromosome counting or flow cytometry (i.e. by significant increase of relative DNA content), we cannot rule out their presence in some individuals. Studies on other plant groups reveal an effect of B chromosomes on increasing genome size (e.g. Rosato et al. 1998, Chumová et al. 2016, Fourastié et al. 2016). However, considering the generally smaller size of B chromosomes and their unequal distribution in cells of the same individual (D'Ambrosio et al. 2017, Bednářová et al. 2021), their presence may not always increase genome size enough for it to be detectable by flow cytometry.

The relative DNA content and its reliability for identifying Solidago ×niederederi

Some previous studies used flow cytometry for ploidy level determination to identify *Solidago* taxa (Verloove et al. 2017) or to map the distribution of cytotypes of polyploid complexes such as *S. altissima* L. and *S. gigantea* (Halverson et al. 2008, Schlaepfer et al. 2008a, Etterson et al. 2016). Variation in genome size is rarely used to differentiate *Solidago* taxa that do not differ in ploidy level (Szymura et al. 2015, Nardi et al. 2018). In the present study, we showed that DAPI flow cytometry is suitable for revealing differences in genome size between diploid *Solidago* species and, moreover, for detecting their hybrids. The relative values of the DNA content of the diploid taxa studied were very close but did not overlap, and all plants analysed could be unambiguously assigned to either *S. canadensis*, *S. ×niederederi* or *S. virgaurea,* based on these values (Table 2, Fig. 4D, E, 5; Supplementary Fig. S5). Further, the relative DNA content of hybrid plants was clearly intermediate when compared with parental taxa (Fig. 5). Thus, the present study demonstrated that, besides polymorphisms in ITS sequences, relative DNA content can be reliably used to identify *S. ×niederederi*. In particular, flow cytometry, which combines accuracy with unsurpassed speed and inexpensiveness (Loureiro et al. 2010), can be effectively used to identify *Solidago* plants with unclear morphology, seedlings and non-flowering or sterile plants, or used for detailed screening of mixed populations and progeny of plants from such populations. Morphology and genome size are highly correlated in several homoploid hybridising complexes (Hanušová et al. 2014, Macková et al. 2017, 2018).

The plants of *S. ×niederederi* analysed are likely to be putative F1 hybrids with intermediate relative DNA content between the parental species. However, variation in relative DNA content (Fig. 5C) showed that values for a few *S. canadensis* plants from mixed populations were out of range of the values detected in pure populations, and in fact were very close to the lowest values recorded for *S. ×niederederi*. This pattern might indicate some level of backcrossing with one of the parental taxa (cf. Bureš et al. 2004, Macková

et al. 2017), but this requires further research. A few discrepancies between morphology, ITS polymorphisms and DNA content values are also recorded, which indicates also cases of backcrossing, but the usage of ITS data may be limited here (see below).

In some groups of plants, genome size is correlated with environmental conditions and/or geographical distribution (Pecinka et al. 2006, Kolář et al. 2009, Dušková et al. 2010, Olšavská et al. 2012). However, we recorded little variation in relative DNA content of *S. virgaurea* samples (5.8%) collected over a substantial part of its distribution area (Fig. 1) and over a wide range of altitudes (190–2130 m a.s.l.; Tables 1, 2). Similarly, Nardi et al. (2018) found no significant differences in absolute DNA content between closely related *S. virgaurea* subsp. *virgaurea* and coastal populations attributed to subsp. *litoralis*. Furthermore, we recorded little variation in the datasets of the European populations of the invasive species *S. canadensis* (5.8%) and *S. gigantea* (5.3%; Table 2) studied. Therefore, it is likely that our results are applicable also in other parts of the non-native range of *S. canadensis,* where it may share habitats with *S. virgaurea* s.l.

Previous studies have shown substantial differences in estimates of DNA content, which may be due to different methodological procedures, different intercalary dyes as well as different plant tissues or internal standards used (cf. Doležel et al. 1998, Loureiro et al. 2006, Bennett et al. 2008, Wang et al. 2015). In the case of *S. canadensis*, previous flow cytometry measurements using intercalating fluorochrome propidium iodide (PI) varied from 1.96 pg (Garcia et al. 2013), 2.1 pg (Bai 2012), 2.04 pg (Kubešová et al. 2010), 2.14 pg (Verloove et al. 2017), 2.03–2.21 pg (Szymura et al. 2015), 2.47 pg (Paniego et al. 2019) to 5.87 pg (Guo et al. 2015). The value of 3.13 pg was recorded using mithramycin dye (Galbraith et al. 1983). Similarly, variation in DNA values was previously revealed by PI flow cytometry for *S. virgaurea*: 2.14 pg (Sliwinska & Thiem 2007), 2.14–2.16 pg (Pustahija et al. 2013), 2.26 pg (Temsch et al. 2010b), 2.31–2.33 pg (Nardi et al. 2018), 2.34–2.36 (Szymura et al. 2015) and 2.35 pg (Garcia et al. 2013). A distinct value of 1.77 pg was revealed using Feulgen densitometry (Vidic et al. 2009). Differences in previous results indicate the need to use flow cytometry with caution for identifying *S.* ×*niederederi* as the present study showed that the relative DNA content values of *S.* ×*niederederi* and parental taxa were very close. Therefore, for the identification of hybrid plants of *S.* ×*niederederi* either by DAPI or PI flow cytometry, it is advisable to analyse a reasonable number of plants of the parent species at the same time. The value of the DNA content or the standard/sample ratio of the suspected hybrid itself may not always allow unambiguous identification. Instead, it should be verified that the revealed value(s) is/are intermediate between the values for *S. canadensis* and *S. virgaurea.*

Genetic variation of the Solidago species studied

The ITS region of nrDNA is a high-copy locus prone to concerted evolution (Álvarez & Wendel 2003), but it is widely known that the process of sequence homogenization is imperfect and intragenomic variation may be common even in diploid species (e.g. Weitemier et al. 2015). Therefore, the presence of 2ISPs among several individuals of both *S. canadensis* and *S. virgaurea*, suggesting the presence of multiple nrDNA copy variants within their genomes, is not surprising. Direct Sanger sequencing does not allow exploration of the full intragenomic ITS diversity; instead, molecular cloning or amplicon high-throughput sequencing would give more detailed insights. Nevertheless,

even with direct sequencing, we were able to identify seven species-specific SNPs that unequivocally differentiated *S. canadensis* and *S. virgaurea* and revealed additive patterns in those sites in *S. ×niederederi*. The same seven SNPs were previously used for the identification of *S. ×niederederi* by Pliszko & Zalewska-Gałosz (2016), while Galkina & Vinogradova (2019) identified only four of them and skipping three additional sites located at the 3' end of ITS2 region. Perfect additivity in all the individuals of *S. ×niederederi* examined, with only very few exceptions (see below), indicates that they represent an early hybrid generation (likely F1), which is in accordance with their occurrence at sites together with both parental species.

The genus *Solidago* is known for its complex taxonomy (Semple & Cook 2006) and comprehensive phylogenetic reconstructions are still largely lacking. Recent phylogenetic studies have primarily focused on the origin of polyploids within particular (diploid-) polyploid complexes, such as *S. gigantea* (Schlaepfer et al. 2008b), *S. altissima* (Sakata et al. 2015) and *S. houghtonii* Torr. et A. Gray (Laureto & Barkman 2011) or on the origin of ecotypes within the diploid species complex *S. virgaurea* (Sakaguchi et al. 2018). Laureto & Barkman (2011) also resolved the relationships among 26 North American *Solidago* species and it is noteworthy they revealed that the divergence in their nuclear and chloroplast genomes only partially reflected the morphological differentiation of the species studied. Both molecular markers used in the present study (ITS region of nrDNA, *rpS15-ycf1* spacer of cpDNA) indicated a close relationship between invasive populations of *S. canadensis* and *S. gigantea* (Supplementary Fig. S2, S3), which agrees with previous studies (Schlaepfer et al. 2008b, Laureto & Barkman 2011, Sakata et al. 2015). Here we targeted one of the most variable intergenic spacers of cpDNA (Prince 2015), but it still revealed the presence of haplotypes shared among the species analysed, along with some species-specific ones. Explanations of haplotype sharing reported by previous authors (Schlaepfer et al. 2008b, Sakata et al. 2015) also match our results: either the phylogenetic resolution of cpDNA intergenic spacers, despite their high mutation rate, is insufficient to differentiate between these close relatives, or extensive ancestral variation has been maintained due to rapid radiation during early stages of diversification (cf. Maddison & Knowles 2006, Moreno-Letelier et al. 2013). Our European samples of *S. canadensis* shared two haplotypes with *S. gigantea* (H7, H9), but also the most widespread haplotype (H1) with *S. virgaurea*. Because *S. canadensis* has spread secondarily throughout Europe only since 1870–1900 (Weber 1998), the widespread sharing of the haplotype with *S. virgaurea* is probably not due to introgression/hybridization between these two species, but supports the hypothesis concerning the maintenance of ancestral variation among species of *Solidago*.

Haplotype variation was found to be similar in native *S. virgaurea* (four haplotypes) and invasive *S. canadensis* (five haplotypes). There are no geographic patterns in the distribution of haplotypes, which, along with high intrapopulational variation, is apparently due to outcrossing and efficient seed dispersal in these species.

Who is the mother of Solidago ×niederederi?

In angiosperms, chloroplasts are predominantly inherited maternally (Corriveau & Coleman 1988). In accord with this general hypothesis, a solely maternal inheritance was confirmed by analyses of the chloroplast haplotypes of the offspring of *Solidago*

gigantea (Schlaepfer et al. 2008b). Building on this presumption, analyses of cpDNA could be useful for revealing the maternal parent of *S.* ×*niederederi*. Pliszko & Zalewska-Gałosz (2016), based on an analysis of the chloroplast *rpl*32–*trn*L locus, stated that hybridization between *S. canadensis* and *S. virgaurea* can happen in both directions. However, Galkina & Vinogradova (2019) later noticed that both parents are relatively polymorphic at this locus, and therefore it is not possible to unambiguously answer the question, which is the maternal parent? The results presented here for the *rpS15-ycf1* spacer of cpDNA showed that 10 samples of *S.* ×*niederederi* from five populations shared the species-specific haplotypes H6 and H9 of *S. canadensis*, while two samples of *S.* ×*niederederi* from the same population shared the species-specific haplotype H3 of *S. virgaurea* (Fig. 3, Table 1). Based on these findings, we can infer that hybridization has occurred in both directions, i.e. both *S. canadensis* and *S. virgaurea* have been involved as maternal progenitors of the hybrids.

Does Solidago ×*niederederi backcross with parental species?*

While hybrid plants of *S.* ×*niederederi* could be determined relatively reliably based on intermediate morphological features (Gudžinskas & Žalneravičius 2016, Karpavičienė & Radušienė 2016, Galkina & Vinogradova 2019), or by cytometric (this study) and ITS analyses (Pliszko & Zalewska-Gałosz 2016, Galkina & Vinogradova 2019, this study), the determination of introgressants resulting from further crossing of hybrids and parental taxa is much more challenging. Within our dataset, we identified two types of putative introgressants based on some discrepancies between morphology, ITS polymorphisms and DNA content values. Four plants (I_C) morphologically resembled *S. canadensis* but displayed four additive 2ISPs (out of seven diagnostic ones for the hybrid) in ITS sequences, and the relatively variable values for DNA content are still within the range of variation recorded for *S. canadensis* (Fig. 3, 5, Supplementary Table S2). On the other hand, one non-flowering plant (I_N) morphologically resembled *S.* ×*niederederi* (Supplementary Fig. S1C) but had a homogenized ITS sequence identical to *S. canadensis* and its relative DNA content was intermediate between *S.* ×*niederederi* and *S. canadensis* (Fig. 5). Thus, both the above-mentioned cases could be attributed to the backcrossing of *S.* ×*niederederi* and *S. canadensis*, but with different extents of concerted evolution in the ITS region and possibly also representing backcrossed individuals of different generations. Nevertheless, it is recognized that concerted evolution acting on ITS of nrDNA may be unpredictable and, for instance, also different evolutionary constraints related to the maintenance of secondary structures may be present and affect mutation rates (Álvarez & Wendel 2003). Therefore, the utility of ITS sequences for detecting introgression may be limited and additional evidence for ongoing introgression or backcrosses is needed.

In any case, multiple polytopic origins of *S.* ×*niederederi* (cf. Skokanová et al. 2020b) indicate, no or only a slight reproduction barrier between *S. canadensis* and *S. virgaurea*. It is expected that the reproduction barrier would not play an important role in further crossing of *S.* ×*niederederi* with any of the parental species. Because plants of *S.* ×*niederederi* are visited by many insects, especially *Diptera* and *Hymenoptera*, and produce viable pollen and seeds (Migdałek et al. 2014, Karpavičienė & Radušienė 2016, Pliszko & Kostrakiewicz-Gierałt 2017, 2018), they could be successfully involved in further

crosses either with parental species or with other hybrid plants. Therefore, the monitoring of the progressive transition of mixed populations into introgressed populations or hybrid swarms could be difficult, as it seems that at least plants resulting from backcrossing of *S. canadensis* and *S.* ×*niederederi* are impossible to distinguish from pure *S. canadensis* by means of their morphology and/or genome size.

Solidago ×*niederederi and its potential threats*

The ongoing homoploid hybridization between *S. canadensis* or *S.* ×*niederederi* and native *S. virgaurea*, regardless of the current extent of this process, represents a potential threat to the *S. virgaurea* complex (Skokanová et al. 2020b). This Eurasian species group includes, besides *S. virgaurea* subsp. *virgaurea*, many taxa and ecotypes adapted to local environments, and they are still undergoing speciation (Sakaguchi et al. 2018). Although the taxonomic and genetic diversity of this species complex is insufficiently explored, subsp. *minuta*, subsp. *pineticola*, subsp. *litoralis*, subsp. *macrorrhiza* and subsp. *rupicola* are currently recognized as nominate subspecies in different parts of Europe (Skokanová et al. 2020b). Hybridization between native and non-indigenous species can imperil native taxa in many ways as documented by dozens of studies. The known scenarios include reproductive interference and decline caused by heterospecific pollen (Suárez-Mariño et al. 2019, Zaya et al. 2021), wasteful production of maladaptive hybrids or demographic swamping (Wolf et al. 2001, Prentis et al. 2007), replacement by viable hybrids or genetic swamping (Ottenburghs 2021), genetic erosion (Johnson et al. 2016), reduced vegetative and sexual fitness of native species in contrast to hybrids (Gallego-Tévar et al. 2019), genetic depletion and reduced fitness of native species (Kellner et al. 2012) and, in some cases, extinction of native populations (Rhymer & Simberloff 1996, Buerkle et al. 2003, Todesco et al. 2016).

Although the degree of the negative effect on native species may differ from case to case depending on the frequency of hybridization and hybrid fitness, hybrid viability and fertility, these circumstances can change over time as hybrids evolve and adapt (Sloop et al. 2009, Li et al. 2021). According to current knowledge, the hybrid *Solidago* ×*niederederi* does not spread vegetatively as successfully as *S. canadensis* because of the absence of the long rhizomes typical of the invasive parent. But hybrid plants are very viable and form large clumps with many flowering stems (Pliszko & Kostrakiewicz-Gierałt 2019). Although the proportion of well-developed fruits is low, the seed germination rate is high, as is the production of viable pollen (Migdałek et al. 2014, Karpavičienė & Radušienė 2016, Pliszko & Kostrakiewicz-Gierałt 2017). Moreover, seedlings of the hybrid are less affected by allelopathic compounds of *Solidago* species than its invasive parent *S. canadensis* (Karpavičienė et al. 2019), which can facilitate the establishment of the hybrid even in areas densely covered with *S. canadensis*. Despite the low number of hybrid individuals at localities inhabited by both parents (one, rarely two to ten hybrid plants, exceptionally more, Skokanová et al. 2020b), we cannot rule out that the fitness of the hybrid and its interaction with native *S. virgaurea* populations may change over time.

As hybridization between *S. canadensis* and *S. virgaurea* can occur in both directions (Pliszko & Zalewska-Gałosz 2016, and this study) and backcrossing of the fertile hybrid with at least *S. canadensis* seems probable (plants denoted as I_C and I_N in our results), another aspect that needs to be considered is the flow of genes from native to invasive

species. It is repeatedly documented that introgression of native fitness-increasing alleles and their phenotypic effects further maintained by natural selection, might promote the invasiveness (by increasing its ecological amplitude and facilitating range expansion) of alien species (Ellstrand & Schierenbeck 2000, Currat et al. 2008, Vekemans 2010, Hall 2016). In our case, it is expected that *S. canadensis*, which is already highly invasive, could pick up genes adapted to local conditions (adaptive introgression) from the highly variable native *S. virgaurea*, creating even better-adapted and thus potentially more-invasive phenotypes.

Currently, we can boldly refute the statement that "hybrids … (*Solidago* ×*niederederi*)… do not appear to be common nor be able to persist" (CABI 2021b). Therefore, it is appropriate to stress the need for monitoring the occurrence, spread and behaviour of alien-to-native hybrid *Solidago* ×*niederederi* in order to mitigate its negative effects on the native flora of Europe.

Supplementary materials

Supplementary Fig. S1. – Examples of non-flowering *Solidago* plants collected in the vegetative stage, identified based on their morphology as *S.* ×*niederederi*.

Supplementary Fig. S2. – Genetic variation of the *Solidago* taxa studied revealed by a NeighborNet network based on the ITS sequences of nrDNA.

Supplementary Fig. S3. – Maximum parsimony network of the cpDNA haplotypes (the *rpS15-ycf1* spacer) of the *Solidago* taxa studied.

Supplementary Fig. S4. – Boxplots depicting the relative DNA contents (RSS, 2C values) of the diploid (2n~2x~18) and tetraploid (2n~4x~36) *Solidago* taxa studied.

Supplementary Fig. S5. – Boxplots and circles depicting the relative DNA contents (RSS, 2C values) of the *Solidago* plants and taxa studied in particular mixed populations.

Supplementary Table S1. – GenBank accessions numbers for ITS and *rpS15-ycf1* sequences of the *Solidago* plants analysed.

Supplementary Table S2. – ITS ribotypes of the *Solidago* individuals arranged by assignment to taxa.

Supplementary Table S3. – Polymorphisms in ITS sequences of *Solidago* individuals, arranged by populations.

Supplementary materials are available at www.preslia.cz

Acknowledgements

We thank to two anonymous reviewers for their valuable comments. This study was financially supported by the Scientific Grant Agency VEGA (grant no. 2/0024/19) and by the Slovak Research and Development Agency (grant no. APVV-19-0134).

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Rozdíly v relativním obsahu DNA spolehlivě identifikují *Solidago ×niederederi*, hybrida původního a invazního druhu

Solidago ×niederederi je nothotaxon vzniklý v Evropě křížením severoamerického druhu *S. canadensis* a původního druhu *S. virgaurea*. Rostoucí počet lokalit *S. ×niederederi* vyvolává potřebu monitorovat jeho výskyt, šíření a chování, aby bylo možné předejít případným negativním důsledkům pro původní evropskou flóru. V této studii jsme se proto zaměřili na testování účinnosti DAPI průtokové cytometrie pro detekci hybridních rostlin *S. ×niederederi*. Dále byly použity sekvence ITS (nrDNA) a *rpS15-ycf1* (cpDNA) k potvrzení hybridního původu analyzovaných rostlin a identifikaci mateřského taxonu (přijemce pylových zrn). Sběr materiálu zahrnoval 60 čistých populací *S. canadensis*, *S. gigantea* a *S. virgaurea* a 16 smíšených populací hybridních rostlin *S. ×niederederi* z 6 států střední Evropy a přilehlých oblastí. U všech zkoumaných jedinců *S. canadensis*, *S. ×niederederi* a *S. virgaurea* byl zjištěn výlučně diploidní (2n~2x~18) relativní obsah DNA, hodnoty pro *S. ×niederederi* byly intermediární ve srovnání se *S. canadensis* a *S. virgaurea*. Relativní obsah DNA *S. ×niederederi* se lišil asi jen o 6 % od *S. canadensis* a *S. virgaurea*, hodnoty se ale nepřekrývaly a rozdíly byly statisticky významné. Relativní obsah DNA druhů *S. canadensis* i *S. virgaurea* měl minimální variabilitu, takže průtoková cytometrie může být pro detekci hybridních rostlin použitelná i v jiných oblastech, kde se rodičovské druhy vyskytují společně. Aditivní intraindividuální polymorfismy v sekvencích ITS potvrdily hybridní původ jedinců (s jedinou výjimkou) přiřazených na základě morfologie k *S. ×niederederi*. ITS analýzy také odhalily mezi rostlinami odpovídajícími morfologií a relativním obsahem DNA druhu *S. canadensis* čtyři možné introgresanty mezi *S. ×niederederi* a tímto druhem. Výsledky pro *rpS15-ycf1* ukázaly, že hybridizace pravděpodobně probíhala v obou směrech. Molekulární analýzy ukázaly blízký vztah invazních populací *S. canadensis* a *S. gigantea*. Lze však vyloučit, že se druh *S. gigantea* podílel na vzniku studovaných hybridních jedinců na základě výlučně tetraploidní ploidní úrovně (2n~4x~36) zjištěné pro *S. gigantea*.

How to cite: Skokanová K., Šingliarová B., Španiel S., Mereďa Jr. P., Mártonfiová L. & Zozomová-Lihová J. (2022) Relative DNA content differences reliably identify *Solidago ×niederederi*, a hybrid between native and invasive alien species. – Preslia 94: 183–213.

Preslia, a journal of the Czech Botanical Society

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