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MOLECULAR EVIDENCE FOR THE STATUS OF *BIDENS CONNATA* MUHL. EX WILLD. AND *B. DECIPIENS* WARNST. IN THE OLD AND NEW WORLD

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Abstract

Earlier, we have established that the European blackjack, which in many literary sources is cited as an invasive North American *Bidens connata*, was described by Carl Warnstorf back in 1895 as *B. decipiens* and had a hybrid origin (*B. frondosa* × *B. cernua*). In this study, we continue to compare the genomes of *B. connata* and *B. decipiens* by molecular genetics and cytological methods. The objects are the F_1 offsprings of *B. frondosa*, *B. connata*, and *B. cernua* collected in 2018 from Minnesota and Wisconsin (USA), grown from seeds in the greenhouse conditions of N.V. Tsitsin Main Botanical Garden of Russian Academy of Sciences, as well as samples of *B. decipiens*, *B. frondosa* and *B. cernua* collected from Eastern Europe (Belarus and European Russia). The nucleotide sequences of nuclear (ITS 1–2) and chloroplast (*trn*L–*trn*F and *rpl*32–*trn*L) DNA were studied. Analysis of the ITS 1–2 site showed that *B. connata* individuals of North America are not hybrids. Analysis of the chloroplast DNA regions confirmed that both taxa, *B. connata* and *B. decipiens*, are evolutionarily close to *B. cernua*.

Introduction

Bidens connata Muehl. ex Willd. is a North American species with a natural range from Alaska in the north to Mexico in the south (Strother and Weedon, 2006). At home, this species has high polymorphism, and several of its varieties are described that include *B. connata* var. *ambiversa* Fassett, var. *anomala* Farwell, var. *fallax* (Warnstorf) Sherff, var. *gracilipes* Fernald, var. *inundata* Fernald, var. *petiolata* (Nuttall) Farwell, var. *pinnata* S. Watson, var. *submutica* Fassett (Sherff, 1937, 1962). These varieties differ in color and sculpture of seed wall, and in the shape of leaves and cypselae. In the second half of the XX century, American botanists based on the morphological characters suggested the hybridogenic nature of *B. connata*. They thought that the parental species of *B. connata* were *B. frondosa* L. and *B. cernua* L. (Crowe and Parker, 1981). *Bidens decipiens* Warnst. was described by Carl Warnstorf in 1895 from the European samples, but later on the plants with a set of similar characteristics were defined by European botanists as

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"*B. connata*" and classified as an alien species of North American origin, although significant differences in morphology between European and American individuals were noted (Andreau and Vilà, 2010; Mayorov and Vinogradova, 2013). In current time, *B. decipiens* is recorded as a synonym of *B. connata* in The Plant List and POWO databases (Royal Botanic Gardens Kew sources).

The type specimens collected by Carl Warnstorf for the European Herbarium project were sent out as an exiccatae to the herbaria of Edinburgh (E), Frankfurt (FR) and Charles University in Prague (PRC) (Global Plants, 2019). We studied the morphological characters of *B. decipiens* in Russia previously. We have discovered that characters of that species are transitional between the North American invasive *B. frondosa* L. and the native *B. cernua* L. (Galkina *et al.*, 2015), which may indicate a hybrid origin of *B. decipiens*. Thus, the achenes of *B. decipiens* are covered with two types of hairs – duplex, consisting of two cells (as in *B. frondosa*), and simple multicellular (as in *B. cernua*). In addition, the achenes of *B. decipiens* are tetrahedral, have four spines (as in *B. cernua*), and are covered with warts (as in *B. frondosa*). The heads of *B. decipiens* are similar to those of *B. frondosa* in size and shape, and its leaves are entire, as in *B. cernua*. On the basis of these data, we hypothesized the hybrid origin of *B. decipiens* (Vinogradova and Galkina, 2015).

Our studies of *B. decipiens* samples and its presumed parental species collected in Eastern Europe confirmed the point of view above (Galkina and Vinogradova, 2019). Analysis of the ITS 1–2 and *trn*L – *trn*F nucleotide sequences of European plants made it possible to prove that *B. decipiens* is a hybridogenic taxon, with maternal species *B. cernua*, and the paternal species *B. frondosa* with a high probability (Galkina and Vinogradova, 2019). At the same time, according to the analysis of ISSR fragments, the paternal species (*B. frondosa*) population itself has a high genetic diversity in the secondary distribution range (Vyšniakienė *et al*., 2018).

For the Asteraceae family, hybridogenic activity has also been established within other genera, for example, sunflower hybrids *Helianthus annuus* × *H. tuberosus*. In the case of hybrids, the chromosomes of one of the parents (or portions of these chromosomes) may be lost in favor of the chromosomes of the second parent (Kantar *et al.*, 2014).

The possibility of a hybrid origin of *B. connata* in North America requires further study. If this taxon was indeed a hybrid of *B. frondosa* and *B. cernua*, then it would be impossible to speak unambiguously about the European origin of *B. decipiens*. Also, there would have been an alternative to the introduction of a hybridogenic taxon into Europe. This study aims to compare the genomes of North American *B. connata* and European *B. decipiens* by molecular and cytological methods to confirm the non-identity of these taxa.

Materials and Methods

Plant material

Seeds of *B. frondosa*, *B. connata* and *B. cernua* were collected during an expedition in October 2018 in the states of Minnesota and Wisconsin (USA) in three locations: vicinity of Rochester, Minnesota Arboretum, and irrigation dam in the Spooner (Table 1). The micromorphological features of achenes were studied using a Keyence VHX 1000 E digital electron microscope. To measure morphometric features, a sample of 50 achenes was taken for each collection point.

Achenes collected in the USA without stratification were sown on October 26, 2018 in a warm greenhouse of the MBG RAS. The obtained seedlings were the main material of our research. For comparative analysis, samples of *B. decipiens*, *B. frondosa*, and *B. cernua*, collected from Eastern Europe (Belarus and the European part of Russia), were also used.

Table 1. Samples of the studied taxa of *Bidens* L.

Species	DNA sample no.	GB accession no.			Place and date of collection
		ITS 1–2	*rpl*32–*trn*L	*trn*L–*trn*F	
B. frondosa	fr_A3	MT126645	MT265305	MT150078	Seeds from USA, Minnesota, vicinity of Rochester, 2018. 44.02 N 92.47 W plants were grown in the greenhouse (Moscow), 2019
	fr_A31	MT126646	MT265306	MT150079	Seeds from USA, Wisconsin, Spooner, 2018
	fr_A32	MT126647	MT265307	MT150080	45.84 N 91.88 W plants were grown in the greenhouse (Moscow), 2019
	fr_A11a	MT671434	MT702807	MT702814	Seeds from USA, Wisconsin, Spooner, 2018,
	fr_A11b	MT671435	MT702808	MT702815	Plants were grown in the greenhouse (Moscow), 2020
	fr_A11c	MT671436	MT702809	MT702816	
	fr_A11d	MT671437	–	–	
	fr_5a	MK559780	MT265308	MK575581	Russia, Vladimir oblast, vicinity of Tasinskiy village, 2014, N55.567° E40.172°
	fr_5b	MK559781	MT265309	MK575582	
	fr_10a	MK559783	–	MK575584	Belarus, Dziaržynsk, 2018
	fr_10b	MK559784	MT265310	MK575585	N53.693° E27.165°
B. connata	conA15a	MT671432	MT702805	MT702812	Seeds of plants from Minnesota Arboretum
	conA15b	MT671433	MT702806	MT702813	Plants were grown in the greenhouse (Moscow), 2020
	con_A2	MT126648	MT265311	MT150081	Seeds from USA, Minnesota, vicinity of Rochester, 2018, 44.02 N 92.47 W Plants were grown in the greenhouse (Moscow), 2019
	con_A2-20	–	MT702804	MT702811	Seeds from USA, Minnesota, vicinity of Rochester, 2018, 44.02 N 92.47 W Plants were grown in the greenhouse (Moscow), 2020
	con_A22	MT126649	MT265312	MT150082	Seeds from USA, Minnesota, vicinity of Rochester, 2018, 44.02 N 92.47 W
	con_A23	MT126650	MT265313	MT150083	Plants were grown in the greenhouse (Moscow), 2019
B. decipiens	de_1a	MK559763	MT265314	MK575566	Russia, Kaluga oblast, Milyatinskoe water reservoir, 2013
	de_1b	MK559764	MT265315	MK575567	N54.4914° E34.3393°
	de_4a	MK559774	MT265316	MK575575	Russia, Vladimir oblast, vicinity of Tasinskiy village, 2014 N55.567° E40.172°
	de_4b	MK559775	MT265317	MK575576	
	de_11a	MK559776	MT265318	MK575577	Belarus, Dziaržynsk, 2018
	de_11b	MK559777	MT265319	MK575578	N53.693° E27.165°
B. cernua	cer_A1	MT126651	MT265320	MT150084	Seeds from USA, Minnesota, vicinity of Rochester, 2018, 44.02 N 92.47 W
	cer_A12	MT126652	MT265321	MT150085	Plants were grown in the greenhouse (Moscow), 2019
	cer_A14	–	MT702803	MT702810	Seeds of plants from Minnesota Arboretum
					Plants were grown in the greenhouse (Moscow), 2020
	cer_8a	MK559757	MT265322	MK575561	Russia, Vladimir oblast, vicinity of Tasinskiy village, 2014 N55.567° E40.172°
	cer_8b	MK559758	MT265323	MK575562	
	cer_9a	MK559760	MT265324	MK575563	Belarus, Dziaržynsk, 2018
	cer_9b	MK559761	MT265325	MK575564	N53.693° E27.165°

Molecular data

DNA was extracted from silicagel dried leaves of *Bidens* taxa following the method of Rogers and Bendich (1985). The herbarium specimens of European plants are stored in the Herbarium of the Tsitsin Main Botanical Garden (MHA). PCR was carried out in a DNA Engine Dyad Peltier Thermal Cycler amplifier (Bio-Rad, United States). For the nuclear ribosomal internal transcribed spacer 1–2 (ITS1–2), *nnc18s10* (forward) and *c26A* (reverse) primers with an annealing temperature of 50°C were used. For the chloroplast DNA, primers were used at the annealing temperature from 0.3 to 65°C (Shaw *et al.*, 2007). For the chloroplast locus *rpl*32–*trn*L we used primers *rpl*32 F (forward) and *trn*L UAG (reverse). For the chloroplast locus *trn*L–*trn*F we used primers *c* (forward) and *f* (reverse). Purification of the PCR product for sequencing was carried out in a mixture of ammonium acetate with ethanol. The nucleotide DNA sequences were determined on an automatic sequencer (Syntol).

Analysis of molecular data

Further processing of the nucleotide sequences was carried out in the BioEdit program. Sequences was aligned using ClustalW than modified manually. ITS1–2 and chloroplast regions were analyzed separately. The data were submitted to GenBank (NCBI, 2020), in which these nucleotide sequences can be found by their accession numbers (Table 1). Phylogenetic tree was constructed in the SplitsTree4 program by neighbor-joining algorithm with bootstrap support. Haplotype networks were constructed using TCS.

Chromosome preparations and analysis of chromosomal data

For chromosome preparations the actively growing young plants root were used. Approximately 1.5–2.0 cm long root tips were harvested separately from the plants and immediately pre-treated with a 2 mM aqueous solution of 8-hydroxyquinoline for 4 h at room temperature (RT) in the dark. A 3:1 ethanol/glacial acetic acid (v/v) mix was used for fixation. Meristems of 2 mm length were cut from fixed root tips and digested in a 10 µl enzyme solution (0.5% cellulase Onozuka R-10 (Serva, Germany) and 0.5% pectolyase Y-23 (Seishin Corp., Japan)) in 10 mM citrate buffer (pH=4.9) for 1 h at +37°C. Suspended cells were used for chromosome preparation as described by Kirov *et al.* (2014) with some modifications.

An AxioScope A1 fluorescent microscope (Zeiss) with phase contrast was used to observe chromosome preparations. The metaphase plates were photographed with a monochrome AxioCam 503 Mono camera and visualized using ZEN software (Zeiss). In each experiment, at least 20 mitotic metaphase plates from each plant were analyzed.

Results and Discussion

The morphological diagnostic characteristics of the studied samples are summarized in the Table 2. Analysis of the ITS 1–2 site showed that *B. frondosa* in the primary range has a rather high polymorphism: all three accessions have substitutions that differentiate them from each other. At the same time, the samples from Wisconsin have a high similarity, though, they have many ambiguous readings of the sequence in several positions (Y – C or T, R – A or G, W – A or T, K – G or T). On the chromatograms obtained from sequencing, such readings appear as double peaks (Fig. 1). This indicates their heterozygous origin and a high polymorphism of *B. frondosa* in its natural range.

American specimens of *B. connata* do not have ambiguous readings of the sequence at all, which does not confirm the possibility of their hybrid origin. Moreover, the European *B. decipiens* has ambiguous readings in several alignment positions, while the nucleotides of the putative

parents (*B. frondosa* and *B. cernua*) in these positions, firstly, differ, and secondly, do not have ambiguous readings, which speak in favor of the hybrid origin of this taxon (Fig. 2, Table 3).

The chromosomes of all studied species were small (<5~ μm) and similar in morphology. Chromosome numbers were established for some samples. They were diploid and *2n* = 48 for *B. connata* (sample from Rochester), *B. frondosa* and *B. decipiens*, and *2n* = 24 for *B. cernua*.

Haplotype networks were built (Fig. 3) based on the result of the analysis of the nuclear (ITS 1–2) and chloroplast regions (*rpl*32–*trn*L and *trn*L–*trn*F) of the DNA of all studied samples.

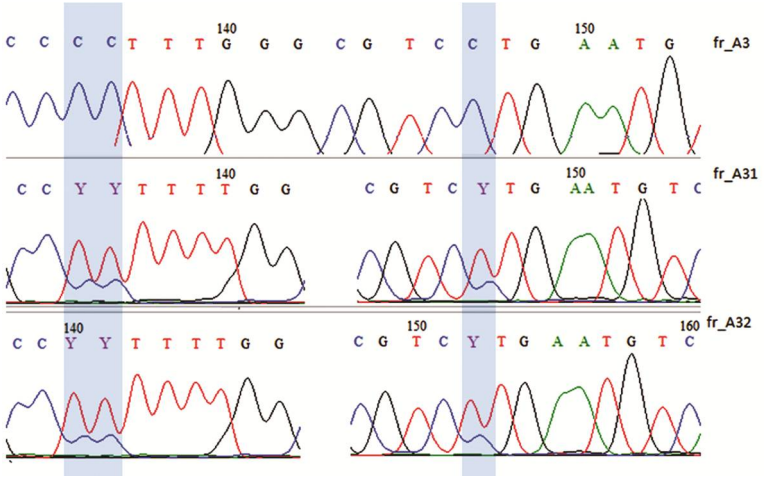

Fig. 1. Fragments of electropherograms of sequences of samples *Bidens frondosa* from North America.

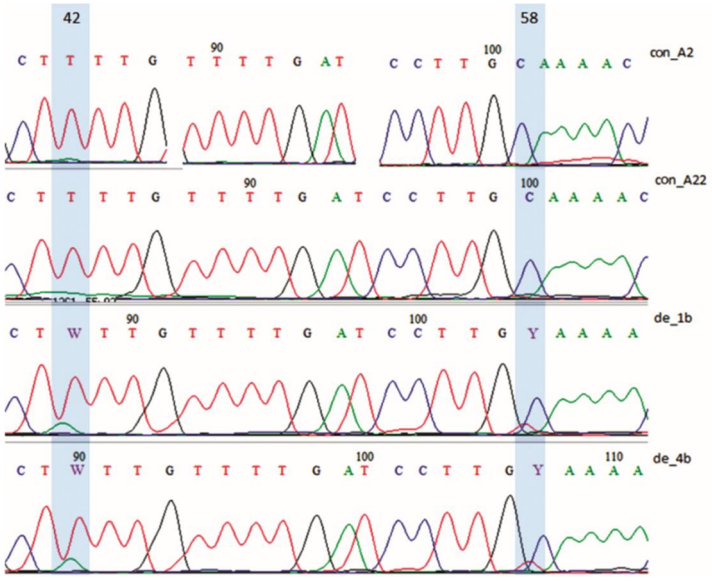

Fig. 2. Fragments of electropherograms of sequences of two samples *Bidens connata* (from North America) and two samples B. *decipiens* (from Eastern Europe). “42” and “58” – number of nucleotide substitutions in Table 3.

As for the ITS 1–2 region, the plants in total formed 7 haplotypes. *B. frondosa* is a very polymorphic taxon, so its all samples were divided into 4 haplotypes, while the common haplotype (№4) included European and American individuals. All *B. connata* samples were assigned to one haplotype along with the majority of *B. cernua* and *B. decipiens* specimens (№1). The haplotypes №2 and №3 are very close to haplotype №1 and include some individuals of *B. cernua* (cer_8a and cer_8b) and *B. decipiens* (de_4b).

Table 2. The main diagnostic characteristics of the studied *Bidens* specimens.

Species	Leaf	Head (inflorescence)	Cypselae
B. frondosa	Blades pinnately compound (3–5 petiolate leaflets) 	Heads erect, diameter 7–15 mm, rays 0	Cypselae with 2 awns, tuberculate, 5,08±0,15 × 1,82±0,07 
			Cypselae with 2 awns, tuberculate, 8,40±0,19 × 3,17±0,07 
B. cernua	Leaves sessile, blades simple 	Heads large (diameter 11–20 mm) and droop, rays 6–8	Cypselae with 4 awns, non-tuberculate, 3,83±0,04 × 1,54±0,02 mm 
B. connata	Leaves petiolulate, blades simple or margins coarsely incised (lobes 3–5) 	Heads erect, diameter 8–13 mm, rays 0–5	Cypselae with 4 awns, tuberculate, 5,60±0,17 × 2,41±0,09 mm 
B. decipiens	Leaves simple, petiolulate, margins dentate or serrate 	Heads erect, diameter 8–13 mm, rays 0	Cypselae with 2-4 awns, tuberculate, 5,32±0,16 × 2,38±0,08 mm 

Regarding the chloroplast site, three similar haplotypes (№ 1, 2, and 3) can be distinguished, which included individuals of *B. cernua* and *B. decipiens*, as well as some samples of *B. connata*. Other individuals of *B. connata* (conA_15a, conA_15b, conA_22, conA_23) formed two separate closely related haplotypes (№ 4 and 5) and turned out to be significantly closer to *B. frondosa* than to *B. cernua* and European *B. decipiens*. For example, in the phylogenetic tree constructed using method of the neighbor-joining, all of *B. connata* samples were divided into two clades – some formed a separate clade (with a bootstrap support of 94%), while others were placed together with all specimens of *B. cernua* and *B. decipiens* with 100% bootstrap support (Fig. 4). We did not build the tree using algorithms that assume the use of an external group. In this case, it would have been preferable to take as an external group a close species growing both in the Old World and in the New World, which was not possible.

Almost all *B. frondosa* samples were included in one haplotype, except fr_A3, which formed a separate haplotype also in the analysis of the nuclear region, and also emerged from the general clade in phylogenetic tree based on chloroplast regions (Figs. 3-4).

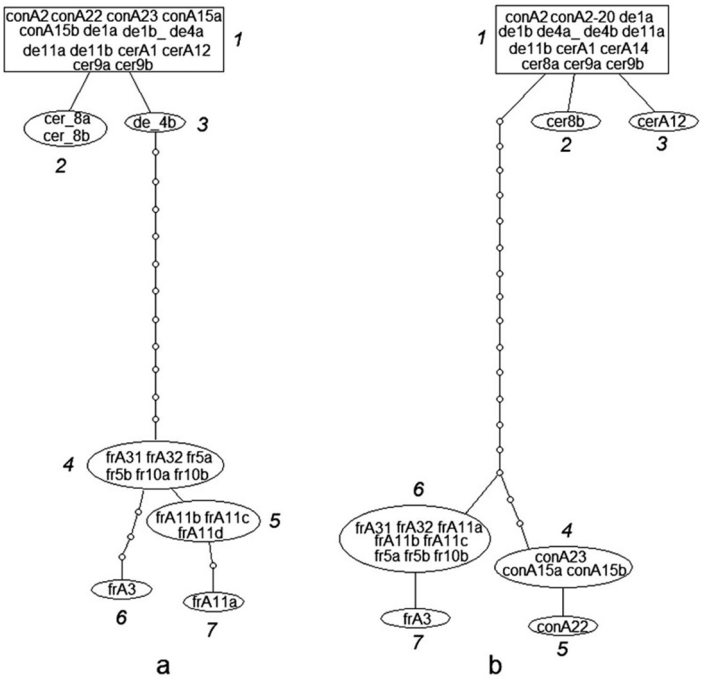

Fig. 3. Haplotype network of different *Bidens* taxa from North America and Europe, a – ITS 1–2, b – chloroplast (*rpl*32–*trn*L and *trn*L–*trn*F). cer = *B. cernua*, con = *B. connata*, de = *B. decipiens*, fr = *B. frondosa*.

The individuals of *B. connata* (neither close to *B. cernua* and European *B. decipiens*, nor close to *B. frondosa* in chloroplast regions) do not have ambiguous readings of the sequence. And not only in the positions differentiating *B. frondosa* and *B. cernua*, but also in others, as, for example, in some American specimens of *B. frondosa*. Analysis of the ITS region does not even confirm the heterozygous origin of American *B. connata* plants. Therefore we cannot establish their hybrid origin.

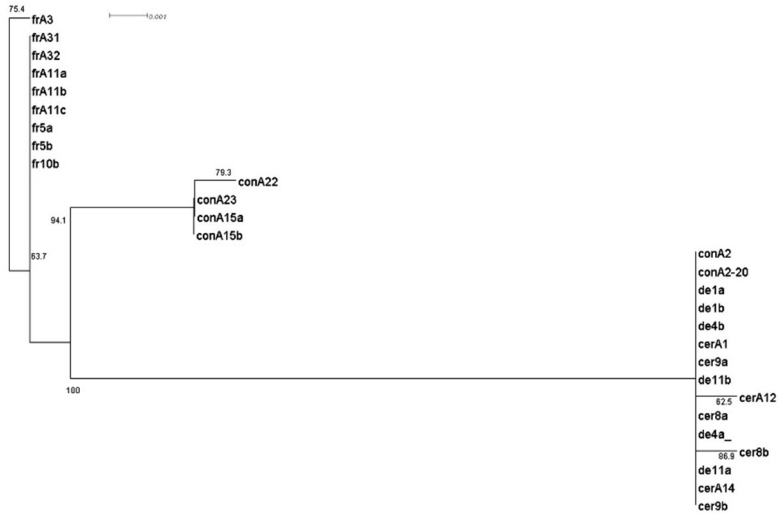

Fig. 4. The Neighbor Joining tree of *Bidens* taxa from North America and Europe based on *rpl*32–*trn*L and *trn*L–*trn*F data. cer = *B. cernua*, con = *B. connata*, de = *B. decipiens*, fr = *B. frondosa*.

Table 3. *B. connata* and *B. decipiens* and its putative parents polymorphism in the ITS 1–2 sequences.

Sample №	Position in the alignment						
	28	42	58	76	90-94	196	425
fr_A3	C	T	T	T	CC	A	G
fr_A31	C	A	T	T	YY	C	R
fr_A32	C	A	T	T	YY	C	R
fr_A11a	C	A	T	T	YY	M	A
fr_A11b	C	A	T	T	YY	M	A
fr_A11c	C	A	T	T	YY	M	A
fr_A11d	C	A	T	T	YY	M	A
fr_5a	C	W	T	T	TCTC	M	A
fr_5b	C	W	T	T	TCTC	M	A
fr_10a	C	W	T	T	TCTC	M	A
fr_10b	C	W	T	T	TCTC	M	A
conA15a	T	T	C	C	C	A	G
conA15b	T	T	C	C	C	A	G
con_A2	T	T	C	C	C	A	G
con_A22	T	T	C	C	C	A	G
con_A23	T	T	C	C	C	A	G
de_1a	Y	W	C	C	C	M	G
de_1b	Y	W	Y	Y	C	M	G
de_4a	Y	W	C	C	Y	M	G
de_4b	Y	W	Y	Y	Y	M	G
de_11a	Y	W	Y	C	Y	A	G
de_11b	Y	W	Y	Y	Y	M	R
cer_A1	T	T	C	C	C	A	G
cer_A12	T	T	C	C	C	A	G
cer_8a	T	T	C	C	C	A	G
cer_8b	T	T	C	C	C	A	G
cer_9a	T	T	C	C	C	A	G
cer_9b	T	T	C	C	C	A	G

We could assume that *B. connata* could still get to Europe, and then it immediately entered hybridization process with *B. cernua*, and in almost a century and a half, "pure" *B. connata* did not remain at all. This hypothesis is supported by the fact that we have established only one of the parents of *B. decipiens* – *B. cernua*. The other parent being *B. frondosa* is our assumption with a high probability (Galkina and Vinogradova, 2019). But this hypothesis is contradicted by the fact that in some areas in Russia, *B. decipiens* was found far from roads (both railways and highways), and *B. frondosa* and *B. cernua* also grow in these habitats. Additionally not a single collection of American *B. connata* was recorded from Europe either in the 19th or in the 20th century. In our opinion, if this hypothesis was correct, then there would be at least an isolated finding of plants with the features of *B. connata*. Therefore, we still adhere to the points that *B. decipiens* arose in Europe independently, and the hybridogenic nature of its origin is confirmed. Our study concludes that (i) *B. decipiens*, native to Europe, is of hybrid origin, unlike *B. connata*, native to North America, (ii) Both *B. connata* and *B. frondosa* show high polymorphism in their natural range in North America, (iii) Chloroplast DNA data support two clades within *B. connata*, in one of which *B. decipiens* and *B. cernua* are nested, and (iv) *B. connata*, *B. cernua*, and *B. decipiens* are phylogeneticly close. *B. cernua* is an older species, and *B. connata* separated from it in America, most likely, several centuries ago. Later, in the 19th century, *B. decipiens* (= *B. frondosa* × *B. cernua*) emerged in Europe independently by hybridogenic way. Since the species is a hybrid, its name can be written as *B.* × *decipiens*.

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