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VERIFICATION OF THE SYSTEMATIC POSITION OF CALIFORNIA BROME (*Bromus carinatus* Hook. AND Arn., Poaceae), cv. 'Broma', ON THE BASIS OF ANALYSIS OF ISSR MARKERS

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A b s t r a c t

'Broma' is a grass cultivar belonging to the species *Bromus carinatus*. In the Lists of Agricultural Plant Varieties of the Research Centre for Cultivar Testing (COBORU), it is shown as *Bromus willdenowii* (= *B. catharticus*, *B. unioloides*) (List of Agricultural Plant Varieties 1989-2009), whereas already in 1984 Mirek demonstrated on the basis of morphological analysis that this was a different closely related species – *B. carinatus*.

The aim of the present study was to verify the species affiliation of cv. 'Broma'. The conducted analysis of ISSR molecular markers included representatives of cv. 'Broma" as well as of *B. carinatus* and *B. willdenowii*.

The method used allowed the identification of molecular markers of the above-mentioned taxa. The numerical analysis of the obtained results suggests that cv. 'Broma' should be classified in the species *B. carinatus*, not *B. willdenowii*.

Key words: *Bromus carinatus*, *Bromus willdenowii*, cv. 'Broma', ISSR, molecular markers.

INTRODUCTION

The genus *Bromus* L. is one of the richest in species in the grass family (Poaceae) and for years it has been an object of great interest for evolutionists, taxonomists, and breeders. S t e b b i n s (1981) divided it into seven groups at the rank of subgenus: *Festucaria, Bromus, Stenobromus, Ceratochloa, Neobromus, Nevskiella, Boissiera.* Numerous polyploids and hybrids found in this plant group create difficulties in determining the number of species. This problem largely relates to the subgenus *Ceratochloa*. Morphological and molecular studies of South American species have shown that it is nearly impossible to identify taxa in this group, in spite of the fact that in morphological terms the subgenus *Ceratochloa* is a well-defined taxon, clearly differing from the other subgenera of the genus *Bromus* (M a s s a et al. 2001, 2004). It comprises annual to perennial plants with spikelets strongly laterally flattened. The plants are characterized, among others, by a 3-5-veined lower glume and a 5-9-veined upper glume (M i r e k , 1984).

In Poland two species are found belonging to the subgenus *Ceratochloa*: *B. willdenowii*, classified as an ephemerophyte, and *B. carinatus* which was not recorded in Poland or in Europe until 1984, but already two years later it was recognised to be an ephemerophyte (R o s t a ń s k i and S o w a , 1986-1987) and subsequently classified as a kenophyte (Z a j ą c et al. 1998). The introduction of some varieties of this species into cultivation in Poland has undoubtedly contributed to its quick spread. In 1973 the cultivar 'Una' was included in COBORU's list of varieties, while in 1988 cv. 'Broma' derived from 'Una' (LORR, 1989).

From the very beginning, these cultivars were wrongly identified as *B. unioloides* or *B. catharticus.* These two synonyms are given as the species name, whereas *B. willdenowii* as a synonym (LORR 1973-2009). M i r e k (1984) showed that the brome introduced into cultivation was not *B. willdenowii* = *B. unioloides*, but *B. carinatus.* Due to this divergence, an attempt was made in the present study to verify the systematic affiliation of the cultivar 'Broma' using molecular methods for this purpose.

Bromus willdenowii Kunth. (syn.: *B. catharticus* Vahl, *B. unioloides* Kunth.) (rescue brome) is a perennial grass found in the wild only in South America, with the number of chromosomes 2n=6x=42. M i r e k (1984) gives detailed morphological characteristics of

this species, drawing attention to the fact that its lemma is awnless or rarely has a very short awn (1-2 mm long). He also points out its quite high variation, in particular as far as the hairiness of the lower glumes and their venation are concerned, and the production of cleistogamous flowers distinguished by shorter anthers. *B. willdenowii* is cultivated as a valuable fodder plant both in its native country and in different regions of Central and North America as well as in Europe (Mirek, 1984). It is popular in livestock farming on account of its high fodder quality, productivity, and pasture persistence. This plant gives the best yields on fertile soils with low aluminium content. This species can be encountered along roads and on baulks (Tsvelev, 1970) as well as in other communities where it finds proper conditions for growth, particularly moisture conditions (Bor, 1970). It is a naturalised species in some European countries, while in Poland it is classified as an ephemeral synanthropic plant (Mirek, 1984).

Bromus carinatus Hook. and Arn. is an octoploid (2n = 8x = 56) native to the western part of North America. Its range extends to the south as far as the Andes in Columbia. In its native country, it is found from lowland areas up to the subalpine zone. This species can be encountered in well-lit forests and thickets, in prairie communities, but also on meadows and in wasteland (Sutkowska and Pasierbiński, 2009). *B. carinatus* is an annual or perennial plant which flowers from June to September. It grows up to a height of 120 cm and its culm terminates in a panicle with drooping branches. More than 7 florets are most frequently borne in laterally flattened spikelets. The lemma terminate in a characteristic long awn (5-12 mm) (Mirek, 1984). It produces cleistogamous, sometimes also chasmogamous flowers, which reduces allogamy (Falkowski, 1982). This species has a deep fibrous root system and hence it is drought-resistant. *B. carinatus* originates from climatic conditions similar to those prevailing in our country, therefore it is no wonder that this plant has quickly settled in Poland as well as in other European countries and it still continues to spread (Mirek, 1984; Pasierbiński et al. 2005; Pasierbiński and Błońska, 2007; Sutkowska and Pasierbiński, 2009).

The cultivar 'Una' was registered in 1973, ten years after its acclimatization and breeding had been started. In their description of *B. unioloides* Humb. and Dunth., Góral et al. (1987a) report, among others: "Spikelets are 10-flowered with a long awn…", and further: "Awnedness of seeds causes difficulties in mechanical sowing".

In their descriptions, both *B. unioloides* (Góral et al. 1987a) and cv. 'Una' (ChORR, 1985; Góral et al. 1987b) have been reported to be characterized by spikelets with a long awn, which Mirek (1984) indicates as a trait characteristic of *B. carinatus*, whereas he describes *B. willdenowii* as a plant with an awnless glume or rarely with a very short awn.

The cultivar 'Broma' was derived from cv. 'Una' and it was entered in COBORU's List of Agricultural Plant Varieties in 1988.

MATERIALS AND METHODS

Material for molecular analysis comprised individuals of the species *Bromus carinatus* and *B. willdenowii* from the subgenus *Ceratochloa*, whose seeds came from the USDA collection (Plant Introduction Station, Pullman, USA) (Table 1), as well as individuals of cv. 'Broma' derived from seeds obtained from the company Poznańska Hodowla Roślin Sp. z o.o. Fully developed, healthy (not infected by pathogens and without feeding traces) leaf blades were used for DNA isolation. DNA was isolated using DNAzol Reagent (Invitrogen) in accordance with the protocol given by the manufacturer.

Following DNA isolation, additional DNA purification was carried out by low melting point agarose gel electrophoresis (Invitrogen) in order to remove any impurities that might hinder or prevent DNA amplification. After the separation was completed, the gel was illuminated with UV light, DNA concentration was estimated, and purified DNA blocks were cut out.

ISSR multilocus molecular markers were used in the investigations; they enable polymorphism analysis of the length of unique nuclear DNA sequences separating microsatellite sequence blocks (Tsumara et al. 1996; Stepansky et al. 1999). Six ISSR primers were used in the molecular analysis (Table 2). Amplification was carried out in 25 µl of reaction mixture containing 5µl of properly diluted DNA samples (100 pg), 2.50 µl of 10 × reaction buffer, 0.75 µl of 25 mM $MgCl_2$, 2 µl of 10 mM dNTP, 1 µl of (ISSR) primer, 0.2 µl of Tag DNA polymerase, and 13.55 µl of distilled water. Primer sequences were taken from the paper of Stepansky et al. (1999). The PCR reaction was performed under the following conditions: initial denaturation at 94°C for 5 minutes, 35 amplification cycles: denaturation at 94°C for 30 seconds, primer annealing at 47°C (for ISSR primers 1 and 3) or 44°C (ISSR 2, 4, 6, and 7) for 30 seconds, polymerization at 72°C for 30 seconds. Additional polymerization was carried out at 72°C for 7 minutes.

Electrophoretic separations were performed in 1.5% agarose gel with the addition of ethidium bromide. The images of electrophoretic separations were archived using the gel documentation system Imagemaster VDS (Pharmacia–Amersham) and original computer software Liscap Capture ver. 1.0.

The results obtained by using ISSR primers 1, 4, 6 and 7, which revealed differences between the investigated plant groups (polymorphism of PCR products), were selected for statistical analysis.

In the case of the other primers (ISSR 2 and 3), the banding patterns of particular individuals did not differ from each other. By using the DNA fragment length marker (GrneRuler100 bp, Fermentas) and computer software GeleScan ver. 1.45 ("Kucharczyk Techniki Elektroforetyczne"), it was possible to determine the length of the PCR products obtained. A 0-1 matrix was constructed by accepting the presence (1) or absence (0) of the PCR product as a trait; this matrix was applied to perform numerical analysis by UPGMA (Unweighted Pair Group Method with Arithmetic Average) using Jaccard's distance (S o k a l and M i c h e n e r , 1958) and multivariate Correspondence Analysis (CA) (G r e e n a c r e , 2007).

RESULTS

15 individuals of cv. 'Broma', 25 individuals of *B. carinatus,* and 30 individuals of *B. willdenowii* were subjected to molecular analysis (Table 1). The primers whose products were included in the analysis generated from 10 to 25 PCR products of different length: ISSR 1-25; ISSR 4-10; ISSR 6-21; and ISSR 7-24. The amplification product length was from 140 bp to 870 bp.

The conducted analysis allowed the determination of banding patterns characteristic of the Polish cultivar 'Broma' as well as of *B. carinatus* and *B. willdenowii* (Table 3, Fig. 1). The PCR products that draw attention are those of ISSR 4: 260 and 330 bp, and ISSR 6: 200 bp; they were found in all the individuals investigated. Marker products were also identified; these are products that were found in 100% of individuals analysed in a given species but did not occur in the other groups of individuals (Table 3).

Two clear groups can be seen in the UPGMA-generated dendrogram and in the chart obtained by using CA analysis on the basis of the molecular analysis results (Figs 2, 3). One is composed of *B. willdenowii*, the other one of *B. carinatus.* The first group is divided into two clades, one of which consists of representatives of the populations CTF and CTE, while the other one consists of the populations CTD, CTC, CTB, and CTA belonging to *B. willdenowii*. The second group is also divided into two clades, one of which consists of representatives of the *B. carinatus* populations: CRB, CRC, CRD, and CRE. Cv. 'Broma' is in the second clade, forming a sister group with the CRA population of *B. carinatus* (Figs 2, 3).

Table 1.
List of samples of *Bromus carinatus* and *B. wildenowii* seeds brought from the USDA collection, Plant Introduction Station, Pullman, USA, with their catalogue number and number of individuals analysed.

Species	Sample designation	Catalogue number	Number of individuals analysed
Bromus carinatus	CRA	Pl 232202	5
	CRB	Pl 232205	2
	CRC	Pl 232203	3
	CRD	Pl 232201	2
	CRE	Pl 232204	6
Bromus wildenowii	CTA	Pl 595117	5
	CTB	Pl 595115	5
	CTC	Pl 595119	5
	CTD	Pl 595114	3
	CTE	9054971	3
	CTF	Pl 595116	3

Table 2.
ISSR primers used in molecular analysis (according to S t e p a n s k y et al. 1999)

Primer	Primer sequence
ISSR1	$(TC)_8C$
ISSR2	$(AG)_8T$
ISSR3	$(GGGTG)_3$
ISSR4	$(ATG)_6$
ISSR6	$(AC)_8G$
ISSR7	$(AC)_8T$

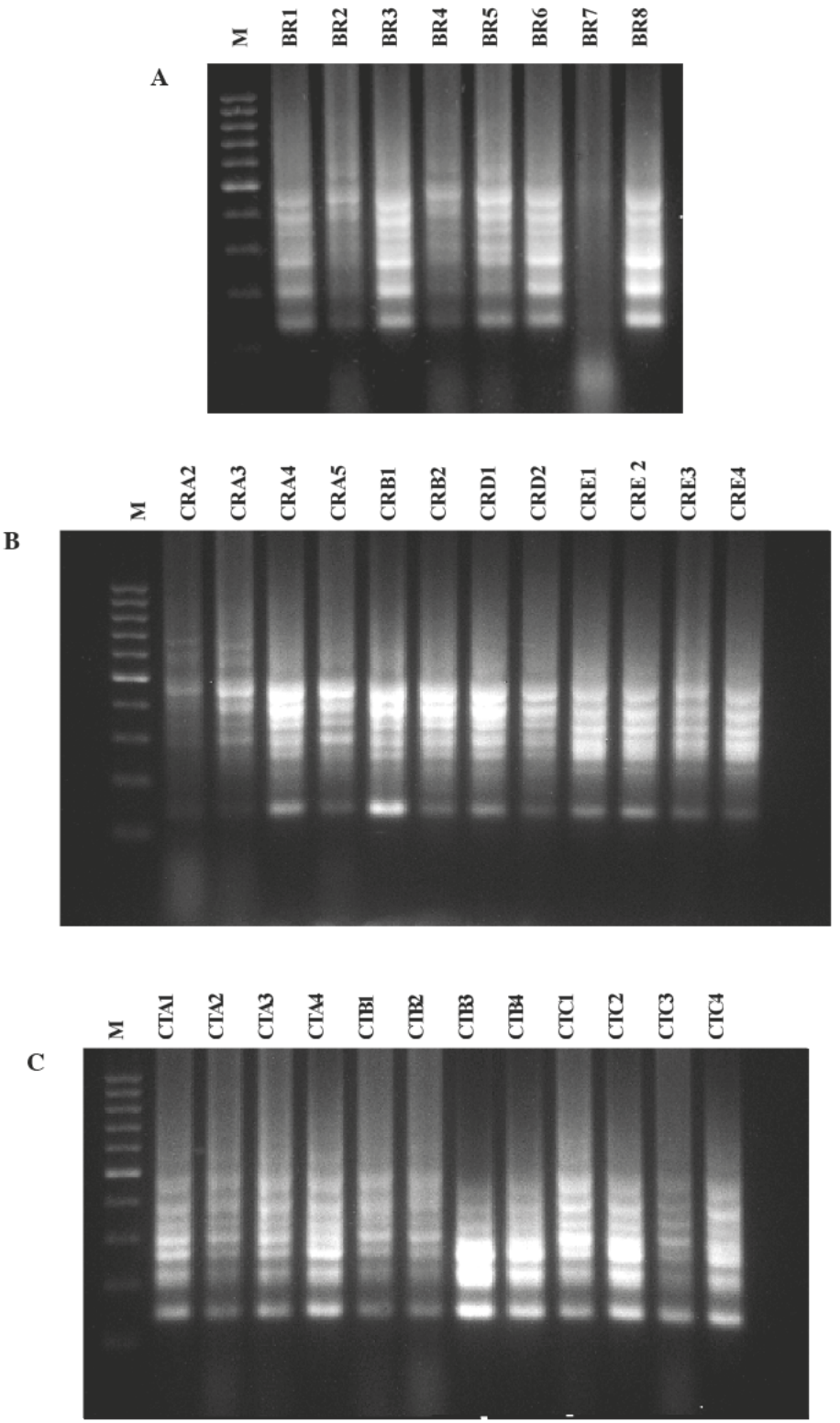

Fig. 1. Example of electrophoretic separation of PCR products generated by using the primer ISSR 6.

A) Cv. 'Broma' – BR1-8;

B) *B. carinatus* – CRA2-5, CRB1-2, CRD1-2, CRE1-4;

C) *B. willdenowii* – CTA1-4, CTB1-4, CTC1-4, M - 100bp DNA fragment length marker.

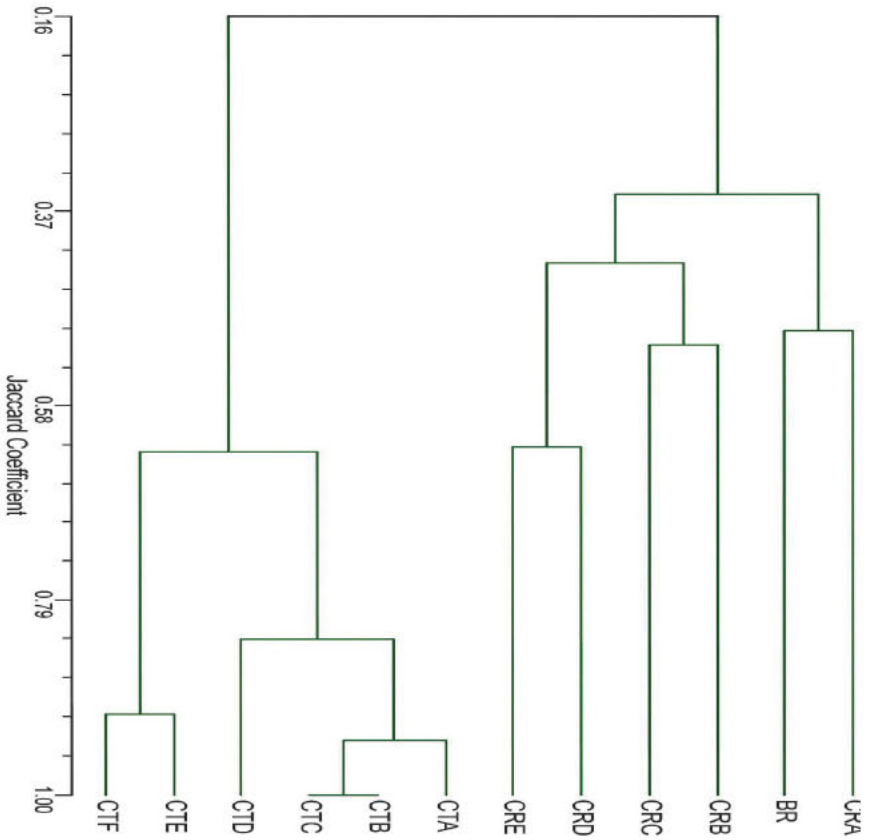

Fig. 2. Numerical analysis of PCR – ISSR products performed by UPGMA: BR – cv. 'Broma'; CRA, CRB, CRC, CRD, CRE – *B. carinatus;* CTA, CTB, CTC, CTD, CTE, CTF – *Bromus willdenowii*.

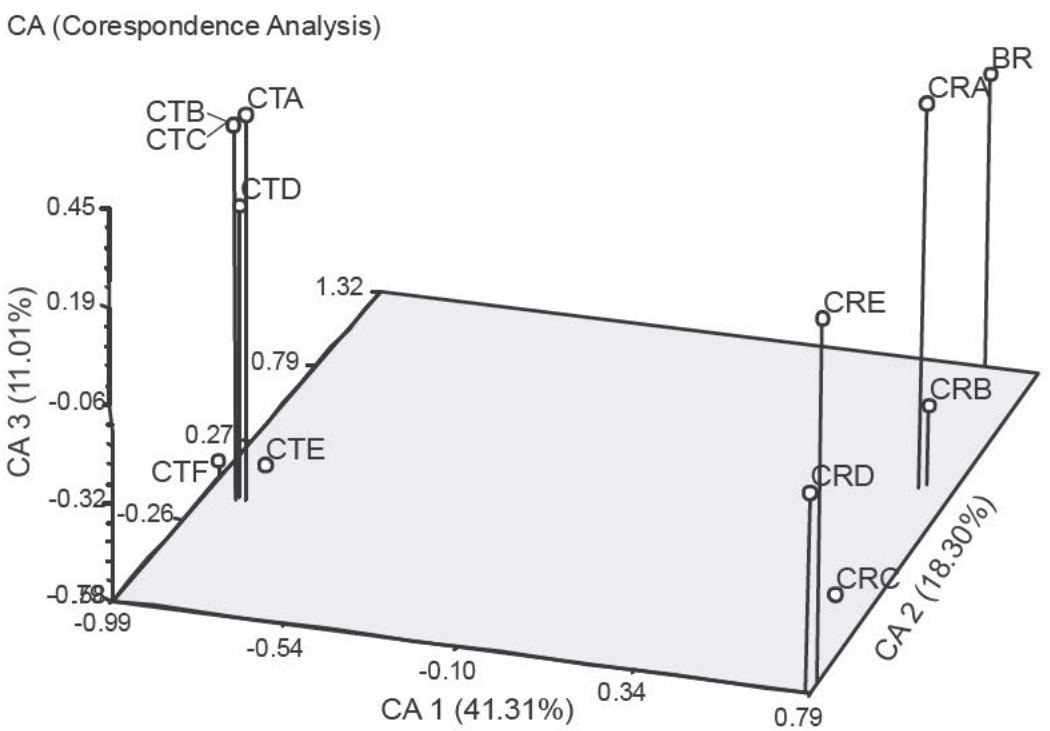

Fig. 3. Numerical analysis of PCR – ISSR products performed by CA: BR – cv. 'Broma'; CRA, CRB, CRC, CRD, CRE – *B. carinatus*; CTA, CTB, CTC, CTD, CTE, CTF – *Bromus willdenowii*.

Table 3.
Marker PCR products for *B. carinatus*, *B. willdenowii,* and cv. 'Broma'.

Primer	Length of PCR products	*Bromus carinatus*	Cv. 'Broma'	*Bromus willdenowii*
ISSR 1	240			
	250		+	
	320			+
	330			
	450		+	+
	700	+	+	
	780		+	
ISSR 4	210	+		
	260	+	+	
	330		+	+
	370			+
	400	+	+	
	540	+		
ISSR 6	140			
	200		+	
	370		+	+
	570	+	+	+
	620		+	
	760		+	
ISSR 7	200			+
	220			+
	250	+	+	
	270			+
	360	+	+	

DISCUSSION

In taxonomic terms, *Bromus carinatus* is one of the most complicated species of the genus *Bromus*. In spite of many studies, its knowledge is still insufficient and this species poses many taxonomic and nomenclature problems. The wrong classification of cv. 'Broma' as the taxon *B. willdenowii* illustrates the problems and gaps in systematics that can be encountered within this genus (K u l a , 1999).

In undertaking the present study, a working hypothesis was made which assumed that PCR products obtained would clearly show the affiliation of cv. 'Broma' with the species *B. carinatus*. The arguments in favour of making such an assumption included the distinct morphological differences between *B. carinatus* and *B. willdenowii* described by M i r e k (1984), the different ploidy levels of both species, the morphological and cytogenetic similarity between cv. 'Broma' and *B. carinatus* as well as the observations made during a previous molecular study on the spread of *B. carinatus* in Poland (S u t k o w s k a and P a s i e r b i ń s k i , 2009).

Nevertheless, the obtained results were not as clear as it had been expected, in spite of the fact that they confirmed the earlier observed absence of variation of cv. 'Broma' and enabled the determination of products specific of the studied taxa.

The molecular analysis performed by using the ISSR–PCR technique determined banding patterns characteristic of cv. 'Broma' as well as of the species *B. willdenowii* and *B. carinatus* originating from the USDA collection. Marker products of the studied taxa were also identified. Products common for cv. 'Broma' and *B. carinatus* as well as PCR products common for cv. Broma, *B. carinatus,* and *B. willdenowii* can be distinguished among them. The analysis of the distribution of the markers between the investigated taxa did not allow us to determine unambiguously in which species cv. 'Broma' should be classified. This results from the close affinity of both species, which was noted by P i l l a y and H i l u (1990, 1995). They found the subgenus *Ceratochloa* to comprise two complexes: *B. carinatus* and *B. willdenowii,* within which all octoploid and hexaploid species had identical chloroplast DNA sequences (cpDNA). These authors also note that the *B. willdenowii* complex must have been the mother component in natural crossing with diploids of the subgenus *Festucaria*, as suggested by S t e b b i n s and T o b y (1944), which led to the creation of *B. carinatus*. Analysing also the banding patterns of *B. carinatus* and *B. willdenowii* by C – banding, high relatedness between these species was shown. Research has identified, among others, six small chromosomes of *B. carinatus* showing affinity with *B. willdenowii* (J o a c h i m i a k et al. 2001).

However, the conducted numerical analysis suggests that the cultivar 'Broma' should be classified as *B. carinatus.* The dendrogram constructed by UPGMA shows a clear division into two clades. One of them groups *B. carinatus* specimens, while the other one comprises *B. willdenowii* individuals. Cv. 'Broma' is in the clade of *B. carinatus*, which undoubtedly defines its species affiliation. Thereby, this confirms the results of the earlier morphological study carried out by Mirek (1984) which showed that cv. 'Una' (from which cv. 'Broma' is derived) introduced into cultivation as a fodder cultivar represented in fact *B. carinatus*, not *B. willdenowii*, as mistakenly reported.

It is also worth stressing that although cpDNA analysis conducted by Pilley and Hilu (1990 and 1995) has significantly enriched our knowledge on hybridizations taking place in the genus *Bromus*, in particular the subgenus *Ceratochloa*, but it has not confirmed the adopted systematic division within this genus. The ISSR-PCR method employed in the present study has made it possible with respect to *B. carinatus* and *B. willdenowii*. The identified markers specific for each species and their clear division into two clades in the numerical analysis prove the divergence of these species.

CONCLUSIONS

The application of the ISSR method allowed the molecular markers of the studied species to be identified. The numerical analysis of the obtained results suggests that cv. 'Broma' should be classified in the species *B. carinatus*, but this requires confirmation by using additional molecular methods.

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Weryfikacja pozycji systematycznej stokłosy spłaszczonej (*Bromus carinatus* Hook. and Arn., Poaceae), cv 'Broma' na podstawie analizy markerów ISSR.

Streszczenie

Broma jest odmianą hodowlaną trawy należącej do gatunku *Bromus carinatus*. Na listach odmian roślin rolniczych Centralnego Ośrodka Badania Odmian Roślin (COBORU) figuruje ona jako *Bromus willdenowii* (=*B. catharticus*, *B. unioloides*) (Lista Odmian Roślin Rolniczych 1989-2009) podczas gdy już w 1984 roku Mirek na podstawie porównawczych badań morfologicznych wykazał, że jest to inny, blisko spokrewniony gatunek – *B. carinatus*.

Celem przeprowadzonych badań była weryfikacja przynależności gatunkowej cv 'Broma'. W przeprowadzonych analizach markerów molekularnych ISSR uwzględniono przedstawicieli cv. 'Broma' oraz *B. carinatus* i *B. willdenowii*.

Zastosowana metoda pozwoliła na identyfikację markerów molekularnych wymienionych taksonów. Analizy numeryczne uzyskanych wyników sugerują, że cv. 'Broma' powinna być zaklasyfikowana do gatunku *B. carinatus*, a nie *B. willdenowii*.