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ADDITIVITY OF ISSR MARKERS IN NATURAL HYBRIDS OF RELATED FOREST SPECIES *BROMUS BENEKENII* AND *B. RAMOSUS* (POACEAE)

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The co-occurrence of hybrids and parental species in similar ecological niches poses a question on the role of traits additivity and overdispersion (emergence of new traits) in microevolutionary processes. We analysed genetic polymorphism of *Bromus benekenii*, *B. ramosus* and the spontaneous hybrid *B. benekenii* × *B. ramosus* in sympatric and allopatric parts of the species distribution in Europe, based on non-coding regions of the taxon genomes (ISSR genetic fingerprinting). We tested 68 individuals in 7 populations, including a hybrid population in N France. Altogether 233 polymorphic ISSR bands (loci) were obtained. We found that the parent species were genetically distinct and the hybrids had an additive pattern of ISSR bands found in the putative parental species (NMDS, STRUCTURE); however, there was evidence of introgression towards *B. ramosus* (NEWHYBRIDS, UPGMA classifications, Nei's D genetic distance). *Bromus benekenii* had 72, *B. ramosus* 21 and the hybrids 9 private bands (genetic overdispersion), probably resulting from the rearranged genomes. Based on its low genetic divergence index DW, the hybrid population seems to be at a young age. We argue that in the face of anthropogenic landscape transformations favouring secondary contacts, the hybrids may competitively replace the parental species in sympatric areas.

Key words: Genetic additivity, hybrid zone, introgression, ISSR, Linnaean taxonomy, microevolution.

INTRODUCTION

The biological species concept assumes that reproductive isolation is the single most important factor giving a rank of species to a group of individuals (Arnold, 1997). In line with this concept, hybrid organisms should not be produced. At the same time, hybridization is regarded as one of the most common evolutionary events in plants (Arnold and Hodges, 1995; Arnold, 1997; Mallet, 2007; Abbott et al., 2013). Among other phenomena, hybridization introduces new genetic diversity and thus provides additional opportunities for new adaptations (Archibald et. al., 2004; Arnold, 1997). Seehausen (2004) views hybridization as the basis of adaptive radiation in both plants and animals. Furthermore, hybridization contributes much to the emergence of new species, being a driving force of speciation called by E. Mayr secondary speciation (Nolte and Tautz, 2010). It pertains chiefly to the plant kingdom, where homoploid hybrid speciation is of special significance (Rieseberg, 1977; Buerkle et al., 2000; Ferguson and Sang, 2001; Mallet, 2007) along with the 'triploid bridge' mechanism (Zieliński, 1982; Buerkle et al., 2000; Rieseberg and Willis, 2007; Mallet, 2007). The absence of genetic barriers between species leads to reticulate evolution, e.g. in the genera of *Aconitum* (Mitka et al., 2007), *Aegilops* (Meimberg et al., 2009), *Armeria* (Fuertes et al., 1999), *Paeonia* (Sang et al., 1997), *Potamogeton*

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(Zalewska-Gałosz and Ronikier, 2012) and in the *Ranunculus auricomus* complex (Hörandl et al., 2009). Hybridization is an essential source of genetic variability in angiosperms, as well as in ferns (Charlesworth, 1995; Soltis and Soltis, 2009; Nolte and Tautz, 2010). Humans often play a significant role in the process of hybridization by breaking down habitat barriers (Bleeker and Hurka, 2001; Oklejewicz et al., 2013).

The frequency of occurrence and survivability of taxonomic hybrids is characteristic of particular groups of plants. In many cases, even closely related species with sympatric ranges and similar habitat requirements do not form hybrids. On the one hand, examples of genera which do not form hybrids include *Vicia* (Muratova, 1931), *Allium*, *Sedum* (Arnold, 1997), many species of bryophytes, whereas hybrids between *Vaccinium myrtillus* and *V. vitis-idaea*, although they are formed in nature, are completely sterile (Stace, 1993). On the other hand, there are numerous groups of plants in which hybridization is such a common phenomenon that hybrid individuals are more numerous than the parent species. Additionally, certain hybridizing species groups demonstrate partial or complete interspecific fertility, to the extent that hybrids and parental species cross freely, which leads to the formation of so-called hybrid swarms. This situation can be observed e.g. in *Salix* (Hardig et al., 2000) and *Tilia* (Fromm, 2003). Such hybrid complexes are also found in *Aconitum* (Sutkowska et al., 2013) and, using Linnaean taxonomy, are given the status of nothotaxa (Mitka, 2003).

The *Poaceae* family is a systematic group particularly predestined to break reproductive isolation barriers. In the areas completely dominated by grasses, a marked species diversity is observed, creating extraordinarily advantageous conditions for hybridization. Anemophily and allogamy, which often occur among grasses, are particularly favourable to the formation of a number of hybrid forms (Frey, 2000). Therefore, it is not strange that in the *Poaceae* family some two thousand interspecific hybrids have been documented and the majority of species (ca. 70%) are polyploids, many of which are of hybrid origin (Mizianty, 1995). For the above reasons, the studies of hybridization within this plant group are of particular significance for understanding of speciation and biogeography of grasses.

From the phytogeographical viewpoint, allopatric (natural) hybridization testifies to the changes and development of plant distribution ranges and secondary contacts (Durand et al., 2000). Furthermore, the occurrence of hybrids provides many useful bits of information about the state of the environment. It heralds the existence of a broad spectrum of ecological niches emerging as a result of 'hybridization of habitats' (c.f. Petit et al., 1999). It also indicates the habitats disturbed due to human activities or because of changes in environmental conditions (Stace, 1997; Oklejewicz et al., 2013).

The objective of this paper was to determine the genetic polymorphism of natural hybrids *B. benekenii* × *B. ramosus* shown against the background of putative parents in allopatric areas. We recognized a putative hybrid zone *B. ramosus* and *B. benekenii* in northern France during our previous studies (Sutkowska et al., 2013). It is formed by two polyploid species of hybridogenous origin: allotetraploid *Bromus benekenii* and allohexaploid *B. ramosus*. It is a case of secondary hybridization. Hybrid zones between cytotypes with different ploidy levels are especially interesting because they may show mechanisms involved in the early stages of polyploid speciation (De Hert et al., 2011; Aagaard et al., 2005). There are no reports on natural hybrids between tetra- and hexaploid species in *Bromus* subgenus *Festucaria* and our studies are the first in this respect.

The analyses covered the non-coding areas of DNA associated with microsatellites of parent species and hybrids, i.e. those indicated by Feldman (1997) as most often subjected to changes during hybridization. When selecting the molecular markers to be studied we paid attention not only to them revealing historical associations between parent taxa but also showing the present structure of the studied genomes. These requirements were met by ISSR markers showing the polymorphism of non-coding areas associated with microsatellite sequences and those located between them. It enables obtaining – at the same time – information on the presence or absence of microsatellite sequences, as well as on the polymorphism of the length of the sequence between them. It is of essential importance during the interpretation of the presented results (see Discussion section). Exactly the same markers were used in studies conducted by other authors examining artificially obtained hybrids (for example Carvalho et al., 2005, Bianco et al., 2011; Grądzielewska et al., 2012; Warzecha et al., 2014), and natural hybrid zones, e.g. in the genus *Aconitum* (Sutkowska et al., 2013), *Spartina* (Baumel et al., 2003) or *Zaluzianskya* (Archibald et al., 2004).

The objectives of our studies were: 1) verifying the existence of the hybrid population *B. ramosus* × *B. benekenii* previously found on the basis of a taxonomic criterion, 2) finding out whether the hybrids are genetically intermediate between pure species originating from the allopatric parts of their geographical ranges, 3) comparing genetic variability in parent forms with that of hybrids. The undertaken studies are the first ever focusing on the natural hybridization between *B. ramosus* and

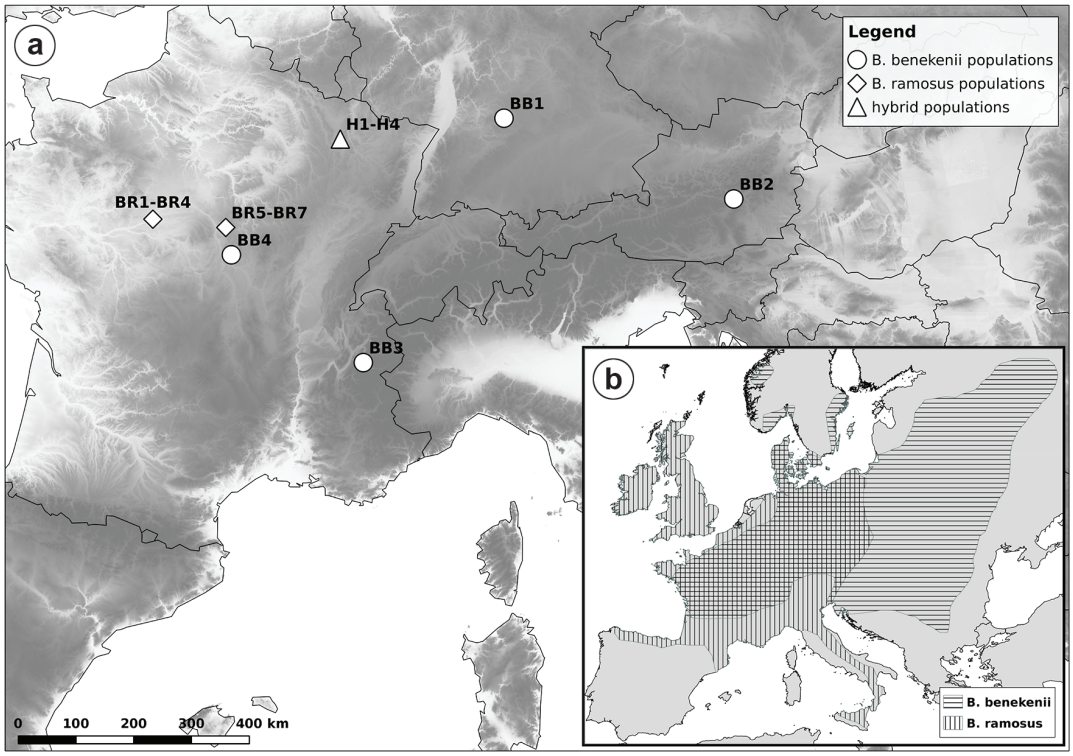

Fig. 1. Localities (**a**) of *Bromus benekenii* (BR) *B. ramosus* (BR) and their hybrids (H) under study (see also Tab. 1), (**b**) Geographical ranges of *B. benekenii* and *B. ramosus* in Europe according to Meusel et al. (1965).

B. benekenii. They also throw some light on hybridization between polyploid species and on differences in the structure of the genomes of parent forms and hybrids arising in natural conditions.

MATERIALS AND METHODS

PLANT MATERIAL

Bromus benekenii (Lange) Trimen and *B. ramosus* Huds. (subg. *Festucaria*) are Eurasian species which – in the flora of Europe – represent relict taxa, phylogenetically linked to species of the New World (they contain the L genome, see Stebbins, 1981; Armstrong, 1984; Sutkowska and Mitka, 2008). They differ in the indumentum of the highest leaf sheath, as well as in the number of branchlets in the lower node of inflorescence. In *Bromus benekenii* the highest leaf sheath is densely, shortly pilose (Fig. S1B), and the number of branchlets is between 3 and 5, whereas in *B. ramosus* the highest leaf sheath is covered by long hairs (Fig. S1A) and the number of branchlets in the lower node of panicle does not exceed two (Bäßler et al., 1994; Rutkowski, 1998). In hybrids these features occur alternatively: a shortly pilose leaf sheath and up to two branchlets in the lower node, or a leaf sheath with long and short hairs (Fig. S1C) and the number of branchlets greater than two. Both species reproduce by allogamy, anemogamy to be more specific (Armstrong, 1984).

These two species differ also in their climatic preferences. *Bromus ramosus* prefers Atlantic climate, whereas *B. benekenii* prefers a more continental climate, though they occur sympatrically over a large area (Fig. 1). However, both species as well as their hybrids have a similar and fairly narrow microhabitat preference. They occur chiefly at forest edges and in lighted fragments of old beech-hornbeam tree stands (beech woods and other broad-leaved forests), they are also characteristic components of clearing communities (alliance *Atropion belladonnae*, Matuszkiewicz, 2001).

The putative hybrids were identified during the studies on *B. benekenii* (Sutkowska et al., 2014) in a population (including four neighbouring sub-populations H1 – H4) in northern France. The material for molecular studies (DNA isolation) consisted of leaf blades of *Bromus* spp. and supposed hybrids (all individuals in the population) collected in the field (Fig. 1, Tab. 1). Molecular examinations were conducted on 68 individuals (Tab. 1). In the neighbourhood of the hybrid population there were no individuals representing parental species.

TABLE 1. List of localities where samples for the study were collected.

Species	Locality	Coordinates		Sub-population denotation	Number of specimens	Habitat description
		Longitude	Latitude			
B. benekenii	Herlebach (Stuttgart, Germany)	9° 48' 24.7"	49° 4' 23"	BB1	4	beech forest
	Sankt Peter-Freienstein (Leoben, Austria)	15° 1' 31"	47° 24' 9.6"	BB2	5	beech forest
	Le Premier Villard (Savoie, France)	6° 15' 25.5"	45° 18' 37.9"	BB3	5	beech forest
	Montigny-aux-Amognes (Nièvre, France)	3° 15' 42.3"	47° 1' 49.5"	BB4	13	beech forest
B. ramosus	Saint-Gervais-la-Forêt (Loir-et-Cher, France)	1° 24' 34.9"	47° 33' 41.5"	BR1	3	hornbeam – beech forest
				BR2	5	hornbeam – beech forest
				BR3	5	hornbeam – beech forest
				BR4	5	hornbeam – beech forest
	Les Coquilliers d'en Bas (Nièvre, France)	3° 7' 15.3"	47° 27' 45.6"	BR5	5	beech forest
				BR6	5	beech forest
				BR7	3	beech forest
Hybrids	Flirey (Meurthe et Moselle, France)	5° 50' 25.2"	48° 51' 52.6"	H1	5	hornbeam – beech forest
				H2	2	hornbeam – beech forest
				H3	1	hornbeam – beech forest
				H4	2	hornbeam – beech forest

DNA ISOLATION AND ISSR ANALYSES

The ISSR (Inter Simple Sequence Repeats) method is based on highly polymorphic sequences of satellite DNA, consisting of a number of nucleotide sequences (microsatellite) tandemly repeated in thousands of copies. This method accesses variation across the genome in the areas around microsatellites (Parker et al., 1998). PCR reaction products are segments of DNA located between the regions which include microsatellite sequences (Stepansky et al., 1999).

The ISSR method is applied to studies of phylogenetic, inter- and intra-population variation and phylogeographic research because utilized primers allow the researcher to identify numerous polymorphic loci in a single PCR reaction without prior knowledge of the genome (Hansen et al., 2000; Li and Ge, 2001; Dangi et al., 2004; Sutkowska et al., 2007; Ilnicki et al., 2011).

DNA was isolated from fully developed leaves without damage symptoms caused by insects or mold. DNA was extracted with a Genomic Mini AX Plant kit (A and A Biotechnology). Primers featuring 2–5 nucleotide repeats (15–18 nucleotides in total) were used. The sequences of primers were taken from Stepansky et al. (1999) and are shown in Tab. 2. Amplification was carried out with 25 μl reaction mixture: 2.5 μl 10-fold concentrated reaction buffer supplied by the Taq DNA polymerase manufacturer (Fermentas), 1.5 mM $MgCl_2$, 0.19 mM of each dNTPs (Fermentas), 27 pmol primer, 100ng template DNA and 1.4 units of Taq polymerase. Reactions were conducted with a 2720 thermal cycler (Applied Biosystems). Annealing temperature for primers ISSR2, ISSR4, ISSR7 was 44°C, and for ISSR1, ISSR3, ISSR5, ISSR6 it was 47°C. Optimal conditions for the reaction were as follows: initial denaturation: 94°C – 5 min, 42 amplificaton cycles: denaturation 94°C – 59 sec, annealing 44°C (47°C) – 59 sec, primer extension 72°C – 59 sec, final extension 72°C – 7 min. A negative control reaction without DNA template was included in each amplification. In order to confirm the results, 50% of the samples were amplified twice.

TABLE 2. The primers used in PCR, primer sequence, total number of reactions products generated by each primer and mean number of PCR product per specimens.

Primer	Primer sequence	Number of PCR products	Number of PCR products per specimen	Mean number of PCR products per specimen
ISSR1	$(TC)_8C$	38	2–9	6
ISSR2	$(AG)_8T$	40	3–12	6
ISSR3	$(GGGTG)_3$	66	2–13	9
ISSR4	$(ATG)_6$	28	3–7	5
ISSR6	$(AC)_8G$	26	4–8	4
ISSR7	$(AC)_8T$	35	2–9	6

The products were subjected to electrophoresis in 1.5% agarose gel stained with ethidium bromide (50 μl/100 ml) at 100V for about 1.5 h. Bands were observed and archived with an Imagemaster VDS (Pharmacia – Amersham). Original software Liscap Capture ver. 1.0 was also applied.

For analysis of band patterns GelScan ver. 1.45 (Kucharczyk TE) software was used. Thanks to the opportunity to create a calibration curve based on the band pattern of markers length (GeneRuler TM 100 bp – Fermentas), it was possible to determine the molecular weight of the resulting amplification products. ISSR reproducibility tests (Bonin et al., 2004) included within-plate (n = 12) and between plate (n = 9) replicates independently analysed from the DNA extracts.

ISSR DATA ANALYSES

At the population level we analyzed PCR-ISSR polymorphism as alleles, under the following assumptions: ISSR products segregate as dominant alleles in Mendelian fashion, genotype frequencies at ISSR loci are in Hardy-Weinberg equilibrium, and comigrating fragments are considered homologous loci (Apostol et al., 1996). Despite the strong assumptions, the high number of ISSR fragments generated from the whole genome can reveal useful discriminatory information for phylogenetic and systematic studies of closely related species (Bussell et al., 2005) and can provide a reliable estimation of genetic variability at the population level (Sica et al., 2005).

The amplification products were coded as binary (0–1) data. The matrix encompassed a total of 233 ISSR interpretable bands obtained from 68 individuals (Tab. 1). The analysis of molecular variance (AMOVA) was based on groups defined by individuals assigned to parental (*Bromus ramosus* BR, *B. benekenii* BB) and hybrid (H) groups. It was based on pairwise square Euclidean distance among individuals. Significance levels were determined based on 1023 permutations. The among-, within- group variation and pairwise F_{ST} values were calculated with ARLEQUIN v3.0 (Excoffier et al., 2005). In order to assign the individuals into predefined genetically homogenous groups, Bayesian algorithm implemented in STRUCTURE v. 2.2 software was used (Pritchard, 2000; Falush, 2007). An admixture ancestry model was used and allele frequencies were correlated; 1000000 replicates of Markov chain Monte Carlo (MCMC) with a burn-in of 20000 iterations gave stable priors and the optimum likelihood for K = 2. The numbers of K from 2 to 5 were tested with ten replicates per each K. In the LOCPRIOR model (Hubisz et al., 2009) the sampling location for each individual was specified, with the expectation that the sampling locations may be informative about ancestry. The STRUCTURE results were analysed with the online software Structure Harvester (Earl and von Holdt 2011; available at http://taylor0.biology.ucla.edu/-structureHarvester/). The likelihood of each run, similarity coefficient among runs (Rosenberg et al., 2002), Evanno's ΔK (Evanno et al., 2005) were computed and visualized. The optimum number of cluster K, was determined on the basis of lnP(D) values and estimates of posterior probabilities plotted as a function of increasing K.

Matrix of Nei's *D* pair-wise genetic distance (after Lynch and Milligan, 1994) was calculated for each pair of individuals with AFLP-SURV (Vekemans, 2002). The matrix was then used to construct a tree, based on an unweighted pair-group method with arithmetic mean (UPGMA) algorithm (Sokal and Michener, 1958). Bootstraps generated in AFLP-SURV based on 1000 runs produced 1000 random trees to obtain a consensus tree plotted with the help of R software (R Development Core Team 2013). Allele frequencies were estimated based on Bayesian method with non-uniform prior distribution of allele frequencies, which efficiently reduces the bias of the square method (Zhivotovsky, 1999). The distribution of allele frequencies was estimated separately for each population. Statistics of genetic diversity and population genetic structure were then

TABLE 3. Genetic diversity within populations and subpopulations of *Bromus* based 233 ISSR bands (loci). BB – *B. benekenii*, H – hybrids, BR – *B. ramosus*.

Group	N	No. loci	*PPB*	Private bands	*I* (S.D.)	*Hj*	S.E.(Hj)	Nei's *D* genetic distance		
								BB	H	BR
BB	29	233	51.9	72	0.317±0.24	0.1529	0.00930	–		
H	10	233	48.1	9	0.240±0.27	0.1457	0.01051	0.0320	–	
BR	31	233	39.9	21	0.231±0.26	0.1268	0.01059	0.0430	0.0220	–

TABLE 4. Analysis of molecular variance (AMOVA) of *Bromus* taxonomic groups (*B. benekenii*, *B. ramosus* and hybrid group).

Source of variation	Sum of squares	Variance components	Percentage variation
Among populations	381.357	8.23198	30.07573***
Within populations	1244.025	19.13885	69.92427
Total	1625.382	27.37082	

*** - $p < 0.001$

computed strictly following the treatment of Lynch and Milligan (1994). Moreover, Nei and Li (1979) distances among individuals were calculated for ISSR data and then used for nonmetric multidimensional scaling analysis (NMDS – Kruskal, 1964). The analysis was performed with the use of the R package *vegan* (R Development Core Team). We estimated the number of private bands, defined as markers found only in one taxon. We calculated the rarity index *DW*, i.e. measure of population genetic divergence, by computing 'frequency-down-weighted marker values' per population and per individual (Schönswetter and Tribsch, 2005). High DW values were expected in long-term isolated populations (Paun et al., 2008). Additionally, the mean number of bands per population FT was calculated. Both indices were calculated only for populations having no individuals with missing ISSR bands. For them one-way ANOVA and *a posteriori* contrasts (Tukey's HSD test) were calculated with STATISTICA ver. 10 (StatSoft, Inc.). Genetic diversity within group of populations was measured by the percentage of polymorphic bands (*PLP*), Nei's (1973) gene diversity or expected heterozygosity (*Hj*) and Shannon's index (*I*). All the indices but Shannon's *I* were calculated with AFLP-SURV, and index *I* with POPGENE ver. 1.32 (Yeh et al., 1999).

To determine hybrid status of individuals, NEWHYBRIDS ver. 1.1 beta Bayesian assignment program, which implements a multilocus allele frequency model-based method (Anderson and Thompson, 2002) was used. This method performs individual clustering without any a priori knowledge of parental allele frequencies, and has the advantage

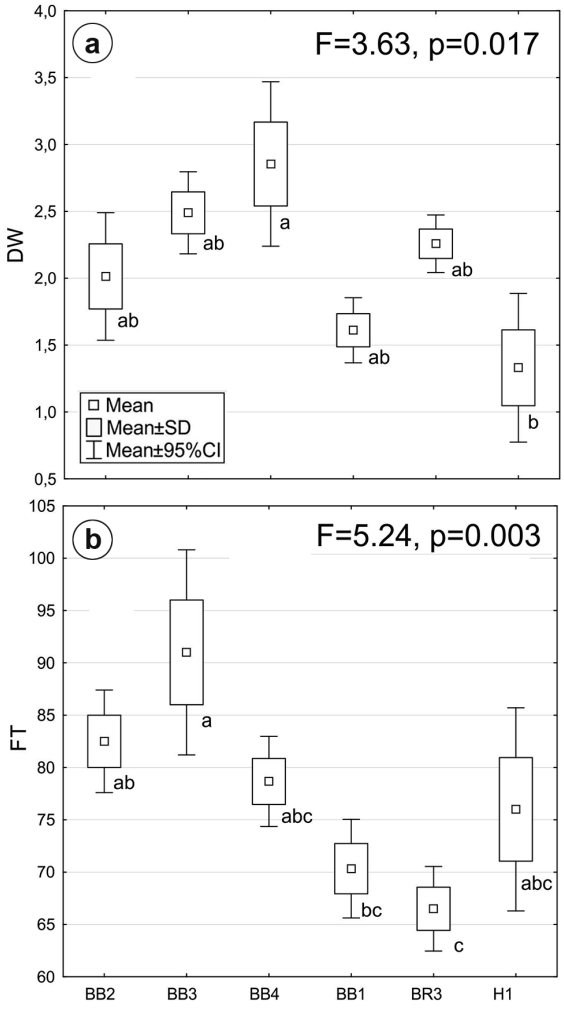

Fig. 2. (**a**) Population divergence index DW, (**b**) Mean number of ISSR bands per population FT in *Bromus* across Europe. ANOVA results and a posteriori contrasts (Tukey's test for unequal sample sizes) are given. The same letter denotes no statistically significant difference ($p<0.05$). The population acronyms are explained in Table 1.

of specifically assuming a mixture of parental and various hybrid classes in its probability model. Six genotype categories corresponding to parental species (*Bromus ramosus* and *B. benekenii*), F1, F2

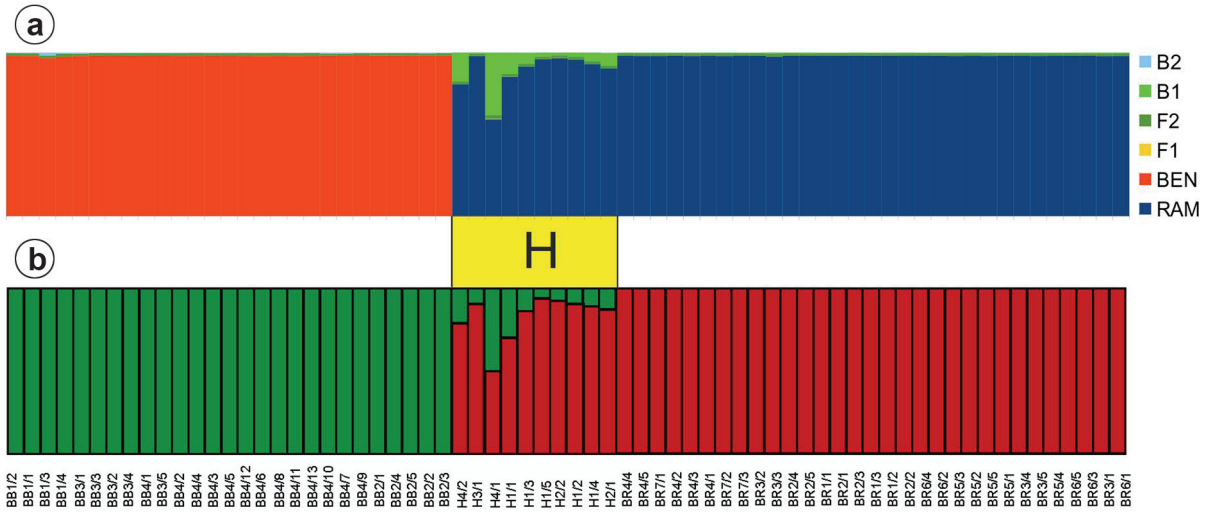

Fig. 3. Diagrams present the results of a Bayesian analysis of *Bromus* population structure in Europe. Each unit represents an individual. (**a**) Classification of *Bromus* individuals according to a Bayesian assignment algorithm (NEWHYBRIDS, Anderson, Thompson, 2002) to detect gene flow. Hybrids (H) were defined as later generation B1 (F1 × *B. ramosus*, see Material and Methods), (**b**) STRUCTURE.

(F1 × F1) and later generation or introgressive hybrids BC1 and BC1b, were defined. The BC1 corresponds to a backcross F1 × *B. ramosus* and $BC1_b$ to a backcross F1 × *B. benekenii*.

Individual posterior probabilities belonging to each hybrid category were estimated using the MCMC method in a Bayesian framework. Calculations were run using Jeffreys-type priors and a burn-in period of 100000 iterations followed by 50000 sweeps for sampling from the posterior distribution.

RESULTS

The genetic diversity indices calculated for the taxonomic groups of *Bromus* showed the intermediate values for the putative hybrid population in percentage polymorphic bands (*PPB*), Shannon's *I* and Nei's gene diversity *Hj*, between *B. ramosus* (the lowest) and *B. benekenii* (the highest genetic diversity, Tab. 3). The Nei's genetic distance value was the biggest between the species of *Bromus* and the smallest between *B. ramosus* and hybrid group. The highest number (72) of the private bands was noted in *B. benekenii*, and the lowest (9) in the hybrid group. *Bromus ramosus* had 21 private bands (Tab. 3). In the AMOVA, the genetic structuring of the taxonomic groups was statistically significant ($p<0.001$), and most of molecular variation (69.92%) was attributable to individuals within groups, and in 30.08% to among-group component (Tab. 4).

Divergence index *DW* in *Bromus* populations was fairly uniform and varied between 1.33 and 2.85. The only significant difference ($p<0.05$) was found between population BB4 (the highest value) and presumably hybrid population H1 (the lowest value, Fig. 2a). The mean number of bands per population *FT* was the highest in BB3 (91) and the lowest in BR3 (66.5). Hybrid population had intermediate *FT* values (Fig. 2b).

In Bayesian groupings both STRUCTURE and NEWHYBRIDS analyses, based on the highest likelihood given to K=2 (Fig. S2a–d, Supplementary Materials), identified main split into the two species: *B. benekenii* and *B. ramosus*, and additional hybrid genetic group consisting of a hybrid taxonomic group *B. benekenii* × *B. ramosus* (Fig. 3a, b). A NEWHYBRIDS software showed the genetic status of each of the individuals: pure *B. benekenii*, pure *B. ramosus*, and later generation hybrids BC1 (F1 × *B. ramosus*), according to a six-class model implemented in the program. One hybrid individual (H3-1) was identified in NEWHYBRIDS as genetically pure *A. ramosus*.

A UPGMA clustering of individuals based on the ISSR band pattern points to coherent genetic structure of both species (support 95–98%, Fig. 4). The hybrid group, highly supported in 99%, was assigned to *B. ramosus* group. A NMDS ordination of *Bromus* individuals clearly showed the separation of *B. benekenii* from *B. ramosus* and intermediate position of the presumed taxonomic hybrid group along the NMDS Axis 1 and its separate position along Axes 2 and 3. One specimen of *B. benekenii* (BB4-3) and

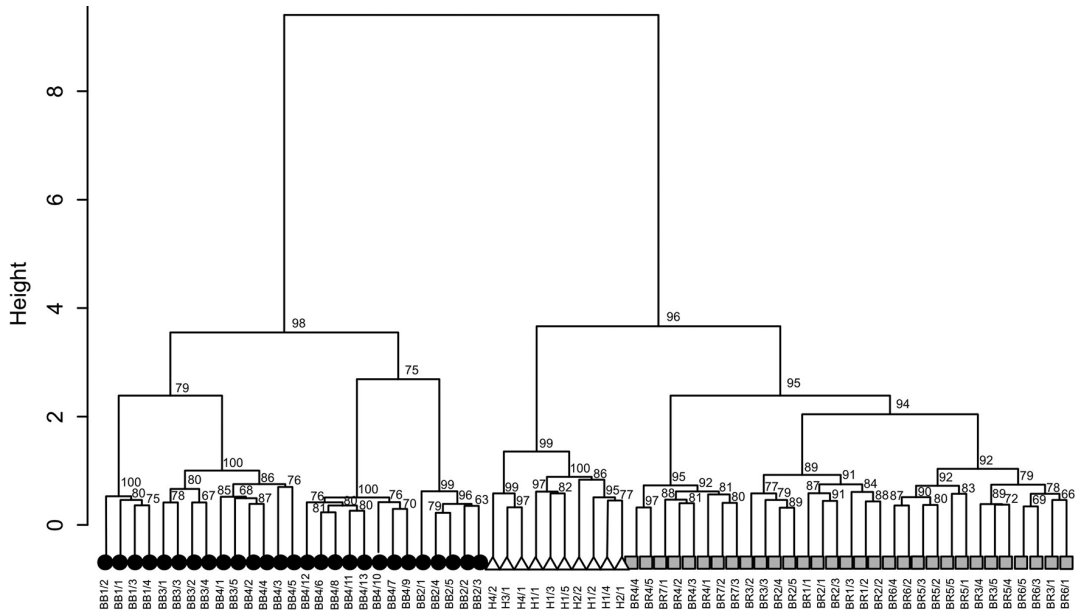

Fig. 4. UPGMA bootstrapped tree (n = 1000) of *Bromus benekenii*, *B. ramosus* and their hybrids collected across Europe based on 233 ISSR bands.

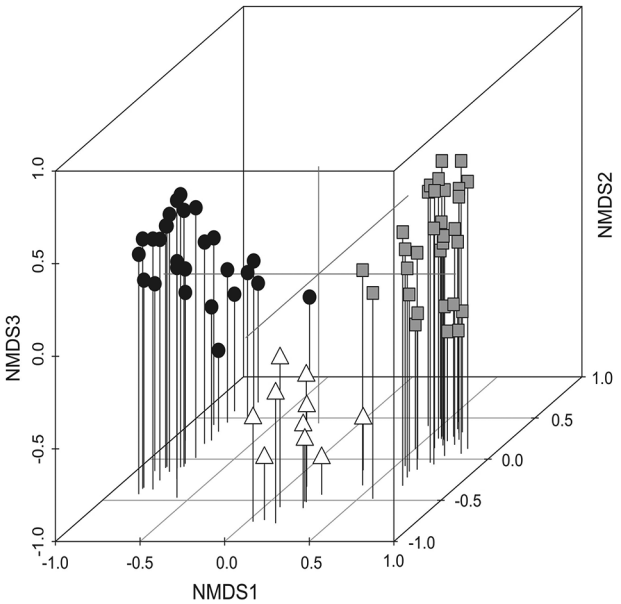

Fig. 5. NMDS ordination of *Bromus benekenii*, *B. ramosus* and their hybrids collected across Europe based on 233 ISSR bands.

two specimens of *B. ramosus* (BR7-2 and BR4-5) gravitated to the hybrid group along the Axis 2 (Fig. 5). Block structure of ISSR bands, based on the UPGMA classifications, showed the intermediate position of the hybrid between the two parental species, resulting from the bands additivity (Fig. S3). In spite of this, 9 ISSR bands were found only in the hybrids.

DISCUSSION

The first objective of the study was to confirm the presumably hybrid nature of individuals collected in natural populations in northern France. The studies confirmed that *Bromus ramosus* and *B. benekenii* should be taxonomically considered as separate species (Sutkowska et al., 2007). On the classification tree, both species form separate, highly supported groups. The hybrid nature of the putatively intermediate population was, however, proved by the multivariate and Bayesian analyses of the molecular data. In both NEWHYBRIDS and STRUCTURE analyses, and on UPGMA clustering and NMDS ordination, the *B. ramosus* × *B. benekenii* population occupied an intermediate position between the parent species or they are linked to the group of *B. ramosus*. It suggests introgression in the direction of *B. ramosus*.

BIOGEOGRAPHY OF THE HYBRID ZONE

As mentioned in the Introduction section, *Bromus ramosus* and *B. benekenii* occupy the same ecological niches and partly overlapping geographical ranges. Here we present the biogeographic hypothesis pertaining to the mechanism of the emergence of the hybrid zone. As shown in our earlier research, in Europe, *B. ramosus* and *B. benekenii* are relict taxa, and they came from a diploid (or diploids) which is/are probably extinct (Sutkowska and Mitka, 2008). The tetraploid *B. benekenii*, problably phylogenetically older than *B. ramosus*, occurred

over a significant area in Europe as early as prior to the last glaciation. It was confirmed by the results of our previous studies proving the post-glacial northbound migration of *B. benekenii* from its refugia in Europe (ranging from the Balkan Peninsula up to eastern France), and the existence of potential cryptic refugia in Central Europe (Sutkowska et al., 2013). *Bromus benekenii*, preferring a more continental climate, extended its wide range in a northerly direction, whereas *B. ramosus*, an Atlantic species, spread in a western direction. Therefore, a sympatric hybrid zone emerged most probably in the Holocene.

Although the area of sympatry of *Bromus ramosus* and *B. benekenii* is wide, hybrid individuals are not found too often. This results from the fact that species occupying similar ecological niches in sympatry undergo strong competition (Gause's Law of competitive exclusion). An idea of reducing competition in Hutchinson's ecological niche ensues that competition leads to an overdispersion of traits and ecological niches in niche-space (Schoener, 1974). The results of this study demonstrate the existence of additive effects in hybrids, combined with the presence of 'private alleles'. The presence of *B. ramosus* × *B. benekenii* hybrids and the absence of parent forms in the studied population ('stranded hybrids') suggest their local competitive superiority and the hybrid's ability to persist where both parental species have become locally extirpated. Thus, the anthropogenic disturbance of habitats, together with postulated genetic (and possibly of traits) overdispersion, can play a chief role in the expansion of hybrids. Also, the climatic changes witnessed at present may be the main driving force behind the emergence of a geographical barrier between *B. ramosus* and *B. benekenii*.

INTROGRESSION

Hybridization in plants is often associated with the fertility of hybrids that enables introgression. Since we have found introgression in the studied specimens, the hybrids must have been fertile and one of the parental form crossed with the hybrid and resulted in accumulation of a larger amount of genetic information of one of the parents. NEWHYBRIDS software demonstrated that in the investigated hybrid population introgression went into *B. ramosus*, where later generation hybrids BC1 (F1 × *B. ramosus*), according to a six-class model implemented in the program, were recognized. BC1 possesses 75% of *Bromus ramosus*. If the F1 hybrids *B. ramosus* × *B. benekenii* were not fertile, we would not get introgression into one of the parent.

Reports on this subject are provided e.g. in studies by Bennuah et al. (2004) pertaining to hybrids between *Picea sitchensis* and *P. glaca* in British Columbia, Leser et al. (2004) on the hybrid zone of *Helianthus*, Shiga and Kadono (2004) on the hybrids between *Nuphar japonica* and *N. pumila* in northern Japan. Our studies found a different degree of genome infiltration in particular individuals. It results from a combination of various factors, of which the different rates of introgression in various areas of genome should be mentioned. For example, Baack and Rieseberg (2007) found that alleles in a certain locus do not undergo introgression, whereas in other loci they transgress freely. This phenomenon depends on the genetic background of species as well as on environmental factors (epigenetic modifications and alterations in gene expression). In the opinion of the above-cited authors, the isolation barriers should not hamper the introgression of neutral alleles (and ISSR markers are one of these), and advantageous ones unless they affect or are correlated with traits contributing to reproductive isolation. The sequences which we analyzed seem to undergo free introgression, because – as they are non coding – they do not affect the adaptation of the studied taxa/hybrids.

In the previous study on natural hybrid zone in *Aconitum* in a sympatric zone in the Carpathians we found some individuals denoted as taxonomically pure species, however designated to the genetic hybrid group in NEWHYBRIDS analysis, a case of cryptic introgression (Sutkowska et. al., 2013). In the present study one specimen, identified as the hybrid on the basis of taxonomic criteria, was genetically recognized as pure *B. ramosus* with the STRUCTURE analytic tool. It means that in some cases a random sample of genetic markers is not representative enough to show the hybridogenous origin of an individual.

VARIABILITY IN PARENT SPECIES AND HYBRIDS

The two studied species differed in the degree of interspecies variability, with *Bromus benekenii* showing much greater variability (*PPB* = 51.9%) at the molecular level than *B. ramosus* (*PPB* = 39.9%). The variability among hybrids assumed values intermediate between the parent taxa (*PPB* = 48.1%). The differences also concern the percentages of 'private bands', i.e. such PCR products which occurred only in one species, and again in *B. benekenii* it had a higher value than in *B. ramosus* (72 and 21, respectively – Tab. 3). The *DW* index, a measure of the population divergence, was the lowest in the hybrid population. The mean number of ISSR bands per population FT was the lowest in *B. ramosus* (BR3) and the hybrid population had intermediate values of the index.

Thus, why do we observe the decline in the variability in hybrids when these new alleles (private bands) can develop in them? First, in the particular

case we studied, one of the parents (*B. ramosus*) is characterised by low variability within the studied area. Even more, the introgression goes in its direction. Therefore, in the genomes of hybrids there is an increase in low-variability DNA (consolidating the low-variability status). Second, the appearance of new alleles does not compensate for the losses from losing parents' alleles (DNA fragments). If a population persists for a sufficiently long time, there may be an increase in its variability via recombinations and random mutations. It probably occurred in another hybrid zone, of *Aconitum lasiocarpum* × *A. variegatum* which we analysed, where hybrid populations differed much in the degree of polymorphism at the DNA level. Some of the studied populations that can be suspected of harbouring in refugia displayed higher polymorphism compared with the newly-developed populations (Sutkowska et al., 2013). However, Nielsen (2000) when studying hybridization in the *Vanilla* genus observed that the variability of hybrids *Vanilla claviculata* × *V. barbellata* was greater than in the parent forms. In the light of the above facts, the low variability of the studied hybrids is indicative of a young age of the analysed hybrid population of *B. ramosus* and *B. benekenii*, supported by the lowest value of the divergence *DW* index.

CONCLUSION

The disturbances of natural systems, chiefly of anthropogenic character (Oklejewicz et al., 2013), as well as the loss of the specificity of ecological niches of species associated with them, favours the emergence of hybrids (hybridization of habitats). Additionally, these changes can be enhanced by phenomena of a random nature. Taking into account the habitat-climatic preferences of *Bromus ramosus* and *B. benekenii*, it is justified to presume that the observed climatic changes can contribute to changes in distribution borders. It can lead to the extension of the hybrid zone. It is not known whether hybridization between the studied species translates into lower viability among hybrid forms. Our research, despite conducted on limited number of specimens, suggests that fully fertile and genetically partly separate hybrids may occupy a separate ecological niche which may lead, after a certain time, to genetic consolidation (parapatric speciation). In the case of disappearance of parent species within a given area, the hybrid may take over their niches. This scenario becomes probable when a shift in the limits of geographical ranges of the parent species is expected. The phenomenon of introgression which we have inferred and the potential rearrangements in the genomes of hybrids (the presence of 'private alleles'), claimed as genetic overdispersion, indicated the viability and adaptive potential of emerging hybrids, therefore the above scenario seems probable.

AUTHORS' CONTRIBUTIONS

AS concept of the article, molecular analysis, interpretation of the results, editing of the manuscript; AP collecting of the specimens in the field, preparation of the maps; WB statistical analysis; TW contribution in molecular analysis, interpretation of some genetic aspects of hybrids origin; JM statistical analysis, interpretation of the statistical analysis results. All authors declare that there are no conflicts of interest.

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