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Diversity and systematics of *Deschampsia* sensu lato (*Poaceae*), inferred from karyotypes, protein electrophoresis, total genomic DNA hybridization and chloroplast DNA analysis

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Abstract: We have analyzed the C-banding karyotypes, electrophoretic mobility of isozymes and seed proteins, total genomic DNA hybridization and chloroplast DNA of *Aristavena setacea*, *Deschampsia cespitosa*, *D. media*, *Avenella flexuosa* and *A. stricta*, which had been previously considered as belonging to the genus *Deschampsia* BEAUV. s. l., in order to determine their taxonomic relationships. The results obtained with these different approaches indicate that *Avenella* diverges substantially in all characters analyzed from the other two genera. No differences in six chloroplast DNA segments analyzed were found between *Aristavena* and *Deschampsia*, which also show a level of total genomic DNA hybridization that indicates a relatively high proportion of common sequences. The closer relationship of these two genera contrasts with their divergence in karyotype structure that suggests a possible allopolyploid origin of *Deschampsia*.

The genus *Deschampsia* BEAUV. sensu lato, belonging to the tribe *Aveneae* (*Gramineae*), involves a group of taxa with a wide geographical distribution which has had a considerably divergent taxonomic treatment.

A large number of previous works (BUSCHMANN 1948; PAUNERO 1955; KAWANO 1963, 1966) emphasized the heterogeneity enclosed in the group, but considered different taxa such as *D. setacea*, *D. cespitosa*, *D. media*, *D. flexuosa* and *D. stricta* within the same genus. However, ALBERS & BUTZIN (1977) disjoined the group in three different genera. The two genera *Aristavena* ALBERS & BUTZIN and *Deschampsia* BEAUV. belonging to subtribe *Aristaveninae* ALBERS & BUTZIN, and the genus *Avenella* DREJER belonging to the subtribe *Airinae* FRIES emend. ALBERS & BUTZIN. In particular, concerning the five taxa previously mentioned, *D. setacea* was included in *Aristavena*, *D. cespitosa* and *D. media* were considered as belonging to genus *Deschampsia* and *D. flexuosa* and *D. stricta* were included in *Avenella*. Although this classification was later supported by means of leaf anatomy, endodermis cells of the

roots, morphology of the spikelets and chromosome number and structure (ALBERS 1980a, b), it seems to be not widely recognized since later works such as CLARKE (1980) maintained *Deschampsia* BEAUV. as the only genus in the group.

In this work, we study the taxa *D. setacea*, *D. cespitosa*, *D. media*, *D. flexuosa* and *D. stricta*, present in northern Spain and Portugal, analyzing their differences in four aspects that can be relevant in the establishment of a more precise systematic treatment of the group: (i) Chromosome number and structure (including C-banding pattern), for which differences between these taxa have previously been described (ALBERS 1980b). (ii) Electrophoretic protein mobility, including isozymes and seed proteins. (iii) Southern hybridization using total genomic DNA, a technique which has been used as a tool for identifying the parents of hybrids and taxonomically closely related species (ANAMTHAWAT —JONSSON & al. 1990, ØRGAARD & HESLOP-HARRISON 1994). (iv) Chloroplast DNA (cpDNA) polymorphisms. Restriction fragment length polymorphism (RFLP) analysis of cpDNA has become a powerful tool for investigating phylogenetic relationships among plant species (LEVÄSLAIHO & al. 1987, PALMER & al. 1988, YANESHITA & al. 1993). Moreover, the design of universal primers for amplification of cpDNA regions by means of the polymerase chain reaction (TABERLET & al. 1991, DEMESURE & al. 1995) enables the use of this technique for evolutionary studies.

Material and methods

Plant material. The plants used in this work were classified according to CLARKE (1980) as *Deschampsia setacea*, *D. cespitosa*, *D. media*, *D. flexuosa* and *D. stricta*, which correspond to *Aristavena setacea*, *Deschampsia cespitosa*, *D. media*, *Avenella flexuosa* and *A. stricta*, respectively, under the classification of ALBERS & BUTZIN (1977). In the remaining of this work, the nomenclature of ALBERS & BUTZIN will be used. The plants were collected in the northern Iberian Peninsula and Portugal (Table 1, Fig. 1), and are maintained in a greenhouse at the Department of Biología Funcional, University of Oviedo.

Karyotype and C-banding analysis. Root tips from the vegetative shoots were immersed in tap water at 0 °C for 24 h to shorten the chromosomes and were fixed in acetic acid-alcohol 1:3. The fixed material was maintained in the fixative for 2–4 months at 4 °C. It was then squashed and stained following the Giemsa C-banding technique described by GIRALDEZ & al. (1979). The karyotypes were made according to chromosome morphology and C-banding pattern.

Isozyme assays. Isozyme analyses were carried out on extracts of leaves from vegetative shoots. Three isozymatic systems, Malate dehydrogenase (MDH, EC 1.1.1.37), Phosphoglucose mutase (PGM, EC 2.7.5.1) and Glucosephosphate isomerase (GPI, EC 5.3.1.9) were assayed after horizontal 12% starch gel electrophoresis using the buffers and staining methods described by FIGUEIRAS & al. (1985) and CHENICEK & HART (1987).

Seed protein assays. 5–10 entire seeds proceeding from the same individual were used for protein extractions following the procedure of ORELLANA & al. (1993). The electrophoresis were assayed in 15% SDS-PAGE gels following the procedure described by LAEMMLI (1970) as modified by PAYNE & al. (1980). The molecular weights of storage proteins were determined from the mobilities of the following proteins included in the MV-SDS-200 Kit (Sigma): bovine albumin (66,000), egg albumin (45,000) and carbonic anhydrase (29,000).

DNA isolation. DNA was extracted from living leaves (200 mg per sample) following the procedure described by SHARP & al. (1988).

Table 1. Taxa, number of samples and origins of the plants studied

Taxa	No. of samples	Location
Aristavena setacea	6	Embalse del Ebro, Corconte, Cantabria, Spain
A. setacea	4	Embalse de Fervenza, Zas, La Coruña, Spain
Deschampsia cespitosa	3	Aguilar de Campoo, Palencia, Spain
D. cespitosa	3	Guardo-Puerto Picones, Palencia, Spain
D. cespitosa	1	Rodrigatos de la Obispalía, León, Spain
D. cespitosa	1	Villafeliz de Babia, León, Spain
D. cespitosa	2	Casares de Arbás, León, Spain
D. cespitosa	4	Cabrillanes-Embalse de B. de Luna, León, Spain
D. cespitosa	4	Pino de Bureba, Burgos, Spain
D. cespitosa	2	Incinillas, Sierra de la Tesla, Burgos, Spain
D. cespitosa	1	Puerto de Leitariegos, Asturias, Spain
D. cespitosa	1	Embalse de Fervenza, Zas, La Coruña, Spain
D. cespitosa	1	La Riva, Cantabria, Spain
D. cespitosa	1	Embalse del Ebro, Corconte, Cantabria, Spain
D. cespitosa	3	Embalse del Ebro, Orzales, Cantabria, Spain
D. media	7	Osona-Seva, Barcelona, Spain
D. media	4	Pino de Bureba, Burgos, Spain
Avenella flexuosa	2	Puerto Ventana, Asturias, Spain
A. flexuosa	1	Puerto Carrales, Burgos, Spain
A. flexuosa	1	Aia-Iturroz, Guipúzcoa, Spain
A. flexuosa	1	Puerto Herrera, Alava, Spain
A. stricta	2	Lagoa Azul, Sierra de Sintra, Portugal

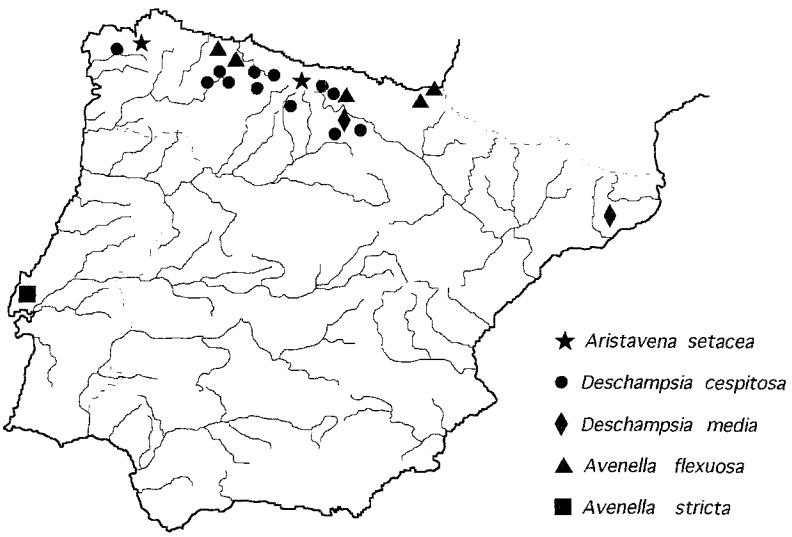

Fig. 1. Collection site of the accessions used in this study

Gel transfers to membranes. Three micrograms of total cellular DNA from each sample was digested with either *Hin*d III or *Dra* I. Restriction fragments were separated by electrophoresis in 1% agarose gel. DNA was transferred to Hybond N nylon membranes. DNA was fixed on the membranes by heating at 80 °C for 2 h. Blots were prehybridized for 4–5 h and hybridized for 16 h at 65 °C in bottles using a Hybaid hybridization oven.

Hybridization with total genomic DNA. For experiments involving total genomic DNA hybridization, DNA fragments of 100–200 bp length were obtained by autoclaving the total genomic DNA for 5 min and used as blocking. Total genomic DNA was [^{32}P]dCTP labeled (DALGLEISH 1987) and used as probe on gel transfers to membrane (SOUTHERN 1975). Different combinations of probe/blocking DNAs from *A. setacea*, *D. cespitosa* and *A. flexuosa* were used in these experiments. In all cases the ratio probe/blocking was 1/200. The blots were washed at high stringency in 2x to 0.1XSSC, 1% SDS at 65 °C. Blots were exposed to Kodak X-Omat films with intensifier screens for 1 h at − 80 °C.

cpDNA restriction fragment analysis. For the characterization of cpDNA the 1.8-kb *Eco* RI/*Hin*d III fragment of *Petunia* cpDNA probe pPCY20-1 (OVERBEEKE & al. 1984) was used. DNA probes were labeled with [^{32}P]dCTP by random priming (DALGLEISH 1987). The blots were washed in 0.2 × SSC, 1% SDS at 65 °C. Blots were exposed to Kodak X-Omat films with intensifier screens for 1 day at − 80 °C.

cpDNA amplification. Five pairs of primers for the amplification of different Chloroplast DNA regions (TABERLET & al. 1991, DEMESURE & al. 1995) were employed (Table 2). The amplification conditions were based on the protocol of TABERLET & al. (1991) with some modifications: PCR reaction mixture (25 µl final volume) contained 50 ng of genomic DNA; dATP, dCTP, dGTP, dTTP each at 200 µM final concentration; 1 µM of each primer; 1x reaction buffer (Boehringer) and 2.5 units of Taq DNA polymerase (Boehringer). DNA amplification was carried out in a Microprocessor Controlled Incubation System (Appligene) programmed for 35 cycles, each consisting of a denaturation step of 1 min at 94 °C, followed by an annealing step of 1 min at 53 °C, and an extension step of 2 min at 72 °C.

The amplification products obtained with primer pairs e/f, g/h and i/j (Table 2) were digested with restriction endonucleases *Bgl* II, *Dra* I, *Hin*d III and *Xba* I.

Amplification products and their restriction endonuclease digestions were loaded and electrophoresed on 1% agarose gel, and detected after staining with ethidium bromide and illumination with UV light. Lambda digests of both *Hin*d III and *Eco*R I were used as size marker in all gels. DNA fragment sizes were determined from digitized images using the NCSA GelReader 2.0 software.

Results

Karyotypes and C-banding pattern. The results concerning the morphology of the chromosomes and their C-banding patterns indicate the existence of three clearly differentiated groups:

(i) *Aristavena setacea* (Fig. 2a) had 2n = 14 acrocentric chromosomes of similar sizes, having small C-heterochromatin bands.

(ii) *Deschampsia cespitosa* (Fig. 2b) and *D. media* had similar karyotypes (2n = 26), being constituted by six pairs of submetacentric chromosomes and seven pairs of acrocentric ones. All chromosomes were similar in size except one of the submetacentric pairs being nearly double in size. The C-banding pattern of most chromosomes is characterized by the presence of a large telomeric C-band in the long arm, a polymorphism for this character being observed in some cases. In some chromosomes, an interstitial C-band was also present.

Table 2. Nucleotide sequences of the primer combinations used in this work and cpDNA regions amplified

Primer pair	Nucleotide sequences	cpDNA region amplified	Reference
a/b	5′CATTACAAATGCGATGCTCT3′/ 5′TCTACCGATTTCGCCATATC3′	trnT(UGU) – trn L(UAA) 5′ exon	TABERLET & al. (1991)
c/d	5′CGAAATCGGTAGACGCTACG3′/ 5′GGGGATAGAGGGACTTGAAC3′	trnL(UAA) 5′ exon – trn L(UAA) 3′ exon	TABERLET & al. (1991)
e/f	5′-ACGGGAATTGAACCCGCGCA-3′ 5′-CCGACTAGTTCCGGGTTCGA-3′	trnH(GUG) – trn K(UUU) exon 1	DEMESURE & al. (1995)
g/h	5′GGGTTGCCCGGGACTCGAAC3′/ 5′CAACGGTAGAGTACTCGGCTTTTA3′	trnK(UUU) exon 1 – trn K(UUU) exon 2	DEMESURE & al. (1995)
i/j	5′GGTCGTGACCAAGAAACCAC3′ 5′GGTTCGAATCCCTCTCTCTC3′	psbC – trnS(UGA)	DEMESURE & al. (1995)

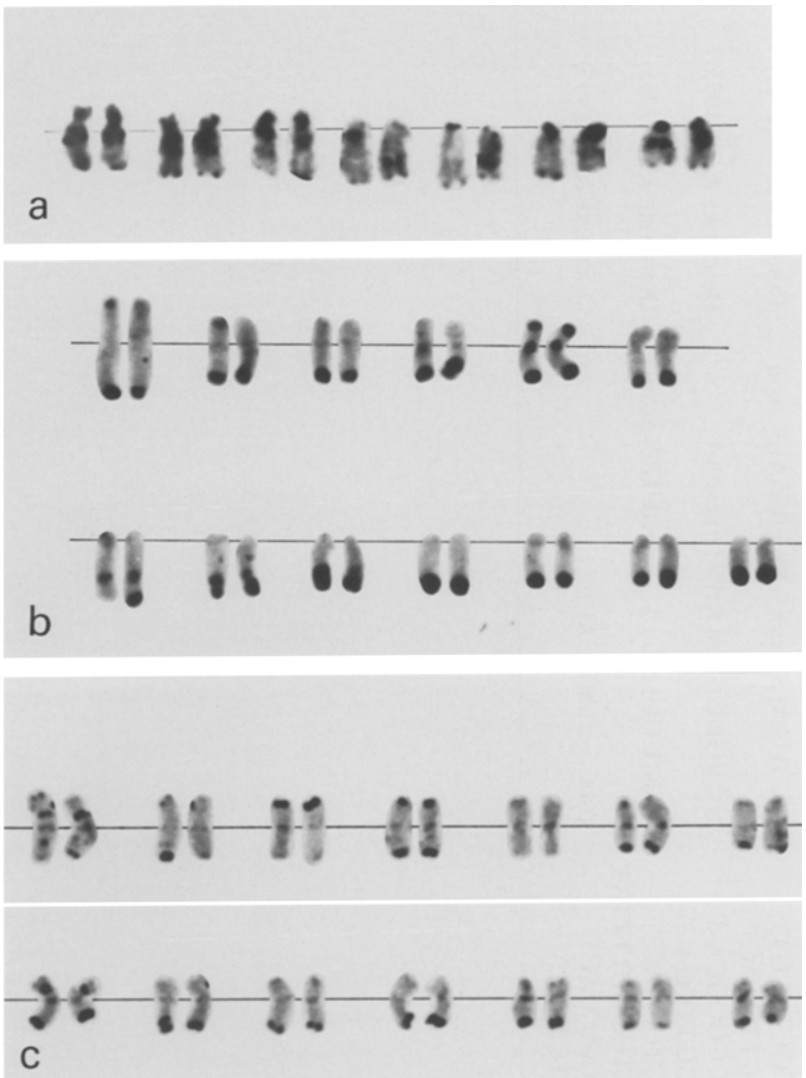

Fig. 2. C-banded karyotypes of *a Aristavena setacea*, *b Deschampsia cespitosa* and *c Avenella flexuosa*

(iii) *Avenella flexuosa* (Fig. 2c) and *A. stricta* had $2n = 28$ and $2n = 56$ submetacentric chromosomes, respectively. In both taxa, all chromosomes were not very different in size. In the individuals analyzed, most C-heterochromatin was forming telomeric C-bands of variable size.

Isozyme assays. The results concerning PGM, PGI and MDH electrophoretic mobilities are shown in Fig. 3. In all taxa analyzed isozyme system PGM showed a unique band, that of *A. setacea* having a lower mobility than those of the remaining species. In GPI isozyme system three different general patterns can be established, *A. setacea* showing a unique band having the highest mobility, *D. media* and *D. cespitosa* showing two or three bands of intermediate mobility and *A. flexuosa* and

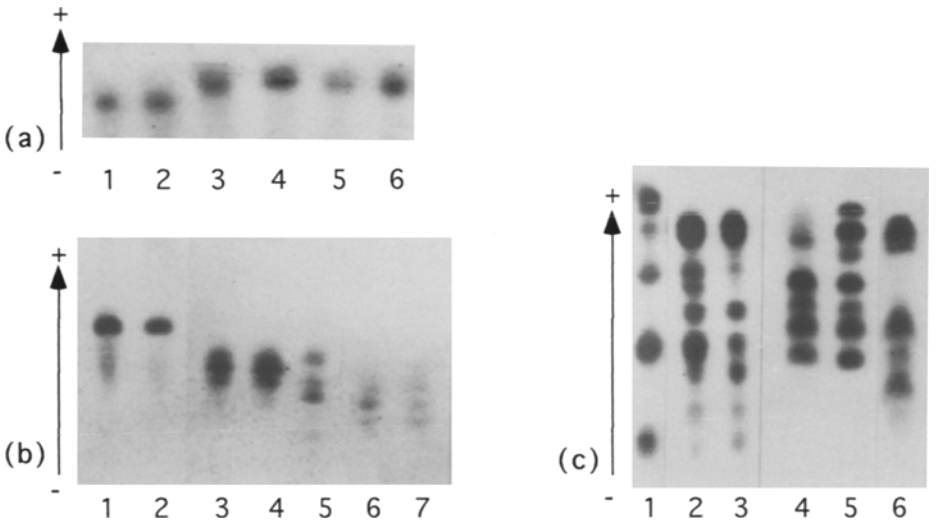

Fig. 3. Electrophoretic patterns of the leaf isozyme systems analyzed. *a* Phosphoglucomutase (PGM; lanes 1 and 2 *Aristavena setacea*, lane 3 *Deschampsia media*, lane 4 *D. cespitosa*, lanes 5 and 6 *Avenella flexuosa*). *b* Glucosephosphate isomerase (GPI; lanes 1 and 2 *Aristavena setacea*, lane 3 *Deschampsia media*, lanes 4 and 5 *D. cespitosa*, lanes 6 and 7 *Avenella flexuosa*). *c* Malate dehydrogenase (MDH; lane 1 *Aristavena setacea*, lane 2 *Deschampsia cespitosa*, lane 3 *D. media*, lanes 4 and 5 *Avenella flexuosa*, lane 6 *A. stricta*)

A. stricta showing three bands in a lower position. In isozyme system MDH, constituted by several bands in all taxa, the general patterns of the taxa analyzed differed from one another, except those of *D. cespitosa* and *D. media* which are very similar. In conclusion the characteristic between species differences in the isozyme patterns correlate with differences in chromosome number and morphology.

Seed proteins. The electrophoretic mobility of seed proteins was analyzed in the three taxa *A. setacea*, *D. cespitosa* and *A. flexuosa* in which seeds were available. The results are shown in Fig. 4. The three taxa analyzed showed different patterns, those of *A. setacea* and *D. cespitosa* showing a higher similarity concerning the number and mobility of the most prominent bands.

Analysis of genomic DNA. A qualitative assessment of the relationship between *Aristavena setacea*, *Deschampsia cespitosa* and *Avenella flexuosa* could be obtained using total genomic DNA. In these experiments isolated DNA from the three species was digested with a restriction endonuclease (*Hin*d III or *Dra* I), electrophoresed and analyzed for the distribution of sequences that hybridize to total DNA of one of the three species, using genomic DNA of the other two species as blocking. The results of the overall hybridization, quantified after 1 h exposure (Fig. 5), indicate a high level of cross-hybridization between *Aristavena setacea* and *Deschampsia cespitosa* and a relatively low similarity between the total genomic DNA of these two taxa and that of *Avenella flexuosa*.

Chloroplast DNA restriction fragments. Fig. 6 shows the restriction fragment patterns of cpDNAs obtained with *Hin*d III and *Dra* I probed with pPCY20-1. The patterns obtained with *Hin*d III were formed by a single band in all cases. *Aristavena*

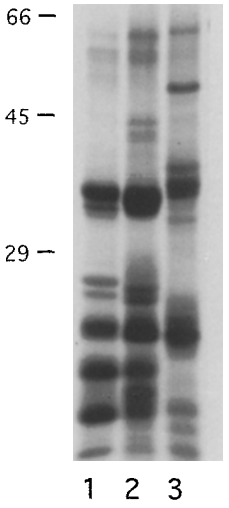

Fig. 4. SDS-PAGE patterns of reduced seed protein extracts of *Aristavena setacea* (lane 1), *Deschampsia cespitosa* (lane 2) and *Avenella flexuosa* (lane 3)

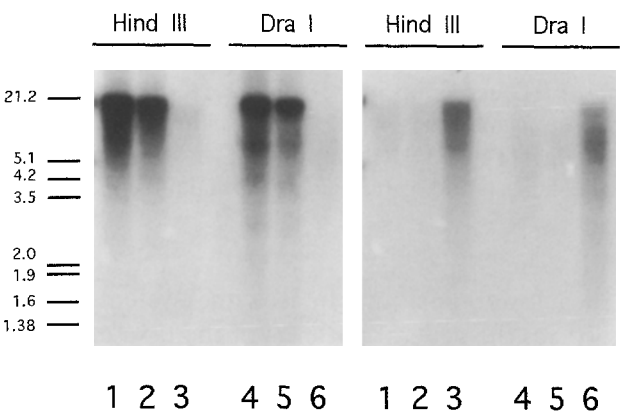

Fig. 5. Total genomic DNA hybridization. *a* Labelled genomic DNA of *Aristavena setacea* hybridized, using genomic DNA of both *Deschampsia cespitosa* and *Avenella flexuosa* as blocking, to *Hind* III and *Dra* I digested genomic DNA from *Aristavena setacea* (lanes 1 and 4), *Deschampsia cespitosa* (lanes 2 and 5) and *Avenella flexuosa* (lanes 3 and 6). *b* Labelled genomic DNA of *Avenella flexuosa* hybridized, using genomic DNA of both *Aristavena setacea* and *Deschampsia cespitosa* as blocking, to *Hind* III and *Dra* I digested genomic DNA from *Aristavena setacea* (lanes 1 and 4), *Deschampsia cespitosa* (lanes 2 and 5) and *Avenella flexuosa* (lanes 3 and 6)

setacea, *Deschampsia media* and *D. cespitosa* showed a similar band of c. 2.1 Kb, while in *Avenella flexuosa* and *A. stricta* a slightly longer fragment was labeled (c. 2.3 Kb). After digestion with *Dra* I, two hybridization bands were found in all taxa analyzed. In *Aristavena setacea*, *Deschampsia media* and *D. cespitosa* the two bands had 3 and 1.6 Kb, whereas in *Avenella flexuosa* and *A. stricta* two labeled bands of 1.9 and 0.85 Kb appeared.

Chloroplast DNA amplification. As it could be expected from the amplification of single-copy region segments of the chloroplast, the five pairs of primers used produced a single band in all cases. The results of amplifications for which between taxa differences were found are shown in Fig. 7. For primer combinations a/b and

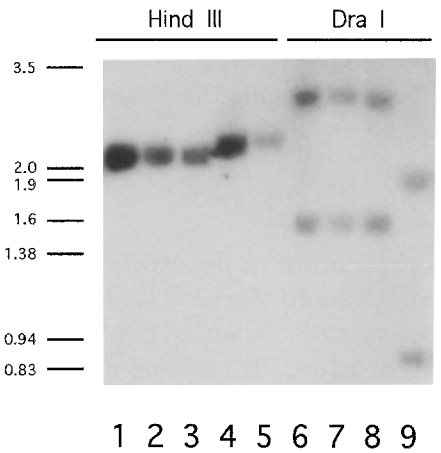

Fig. 6. Hybridization of *Petunia* cpDNA probe pPCY20-1 with *Hin*d III and *Dra* I digests of total DNA of *Aristavena setacea* (lanes 1 and 6), *Deschampsia cespitosa* (lanes 2 and 7), *D. media* (lanes 3 and 8), *Avenella flexuosa* (lanes 4 and 9), and *A. stricta* (lane 5)

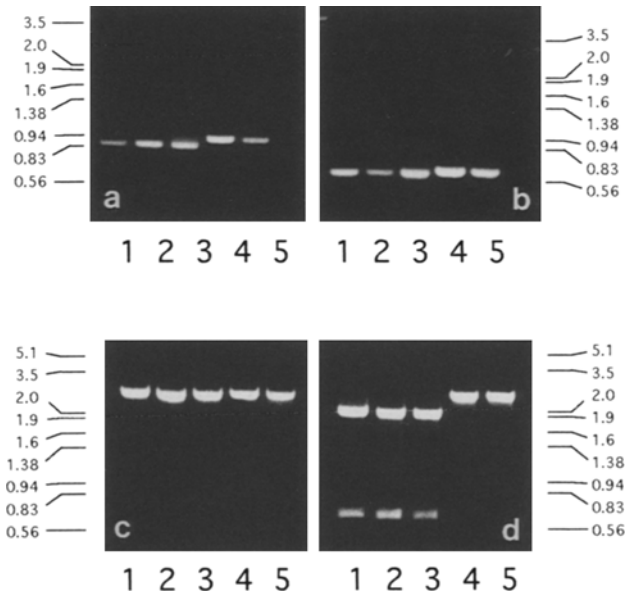

Fig. 7. Gel electrophoresis of the cpDNA amplification products from *Aristavena setacea* (lanes 1), *Deschampsia cespitosa* (lanes 2), *D. media* (lanes 3), *Avenella flexuosa* (lanes 4), and *D. stricta* (lanes 5). *a* Primer pair a/b. *b* Primer pair b/c. *c* Primer pair g/h. *d* Amplification products with primer pair g/h digested with restriction endonuclease *Bgl* II

c/d two types of amplification products could be identified among the five taxa analyzed. *Aristavena setacea*, *Deschampsia cespitosa* and *D. media* showed similar bands of approximately 0.8 Kb, 0.62 Kb with primer pairs a/b and c/d, respectively. *Avenella flexuosa* and *A. stricta* showed longer amplification products in both cases (0.85 Kb with primers a/b and 0.63 Kb with primers c/d).

The size of the amplification product obtained with primer pair g/h was similar in the five taxa analyzed (c. 2.5 Kb). However, differences were found when this

amplification product was digested with the restriction endonuclease *Bgl* II (Fig. 7). *Aristavena setacea*, *Deschampsia media* and *D. cespitosa* have one recognition site for *Bgl* II showing two bands of 1.9 and 0.6 Kb respectively, while the amplification products of *Avenella flexuosa* and *A. stricta* remained uncut.

Discussion

From the data obtained in the four approaches carried out in this work, relative evolutive distances between the taxa analyzed can be deduced. *Avenella flexuosa* and *A. stricta* are very distantly related to *Aristavena setacea*, *Deschampsia cespitosa* and *D. media*, differing in chromosome number and structure, in isozyme and seed protein patterns, and in three out of six cpDNA segments analyzed. Accordingly, a low level of genomic DNA hybridization between *Avenella* and the other two genera was found.

The results concerning cpDNA and total genomic DNA hybridization of *Aristavena* and *Deschampsia* indicate their closer genetic relationship. However, this similarity of overall DNA sequences contrasts with their differing karyotypes. The possibility of an allopolyploid origin for *Deschampsia*, with *Aristavena* (or a close relative) as one of the progenitor species involved, could explain this discrepancy. The karyotype of *Deschampsia*, containing seven acrocentric chromosome pairs and six submetacentric ones, could be generated by allopolyploidy from *Aristavena* (having seven acrocentric chromosome pairs) and another species having a different karyotype constitution. The presence of an almost double sized chromosome pair in *Deschampsia* (Fig. 2b) suggests that it could result from a translocation (or a centric fusion) between two normal sized acrocentric chromosomes, giving rise to the unusual chromosome number of *Deschampsia* ($2n = 26$) when compared to other members of the *Aveneae*.

As a conclusion, in agreement with the proposal given by ALBERS & BUTZIN (1977), the results obtained in this work support the consideration of *Aristavena*, *Deschampsia* and *Avenella* as different genera. Also, the inclusion of these three genera into the subtribes *Aristaveninae* (*Aristavena* and *Deschampsia*) and *Airinae* (*Avenella*) would provide a more appropriate picture of their evolutive relationships.

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