Botanical Journal of the Linnean Society (1997), *124:* 309–318. With 6 figures

Diversity of *Avenella* Drejer (Gramineae) in the Iberian Peninsula inferred from micromorphology, cytogenetic analysis, isozyme assays and *in situ* hybridization

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Received March 1997; accepted for publication June 1997

Micromorphology, cytogenetic analysis, isozyme assays, and *in situ* hybridization were used in order to analyse the diversity of *Avenella* in the north-west Iberian Peninsula. Among the taxa analysed, somatic chromosome numbers $2n=4x=28$ and $2n=8x=56$ were found. Differences in habit, micromorphology and MDH patterns were also detected. Chromosome C-banding pattern and results obtained from total genomic DNA *in situ* hybridization indicate that the different taxa analysed are closely related. The systematic position of the different taxa is analysed.

ADDITIONAL KEY WORDS:—C-banding – FISH – isozymes – micromorphology.

CONTENTS

INTRODUCTION

The taxa considered here to belong to the genus *Avenella* Drejer (Gramineae) are usually included in the genus *Deschampsia* P. Beauv. (García-Suárez *et al.*, 1997). From a morphological point of view they include a rather homogeneous group of

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0024–4074/97/120309+10 $25.00/0/bt970124

plants with a wide geographical distribution that, in relation to Europe, was considered by several authors (e.g. Clarke, 1980) as belonging to a single species [*Deschampsia flexuosa* (L.) Trin.] without any infraspecific division. Nevertheless, Kerguélen (1983) reported that *D. flexuosa* does not constitute a homogeneous group in France. Also, in the Iberian territories these plants show a certain heterogeneity that has led to the description of several taxa with different ranks.

Willkomm (1870) differentiated two varieties from the typical *Deschampsia flexuosa* (β *stricta* Gay ex Willk. from the high Cantabrian mountains and δ *brachyphylla* Gay ex Willk. from the high Cantabrian mountains and Spanish Sistema Central). A new species (*D. stricta*) from the Portuguese mountains of Sintra and Gerez was described by Hackel (1880). Later, Willkomm (1893) added another variety of *D. flexuosa* (var. *orophila*, from Sierra de Palma, Cádiz). More recently, Rivas-Martínez, Izco & Costa (1971) described from Puerto de Navacerrada (Madrid) *D. flexuosa* subsp. *iberica*, a subspecies spread over the Iberian high mountains.

The different systematic studies carried out in this group of grasses have been based on morphological and anatomical characters. More recently, cytogenetic characters have been useful in the determination of the taxonomic relationships between the genera *Aristavena*, *Deschampsia* and *Avenella*, which had been previously considered to belong to the genus *Deschampsia* P. Beauv. s.l. (Albers, 1978, 1980a; García-Suárez *et al.*, 1997).

In the present work we analyse the diversity of the genus *Avenella* in the north-west Iberian Peninsula. Micromorphology and plant habit, cytogenetic analysis, isozyme assays, and *in situ* hybridization were used in order to achieve these aims.

MATERIAL AND METHODS

Plant material

The plants used in this work (Table 1) were collected in the north-west Iberian Peninsula (Fig. 1) and are maintained in a greenhouse at the Department of Biología Funcional, University of Oviedo.

Micromorphology and plant habit

The existence and type of foliar trichomes were examined in epidermal peels directly stripped off the abaxial surface of leaves of vegetative shoots. The epidermal peels were observed by light microscopy. Both micromorphological characters and plant habit were analysed in field samples and in the same plants after a period of cultivation in the greenhouse.

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 1:3 acetic acid-ethanol. The fixed material was kept 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, Cermeño & Orellana (1979). The karyotypes were obtained according to chromosome morphology and C-banding pattern.

TABLE 1. Locality numbers, sample symbols, origin, territories and bioclimatic belts for the *Avenella* plants used in this study

Localities	Samples	Origin	Territory (a)	Bioclimatic belt (b)
		Spain		
1	F5	Guipúzcoa, Aia-Iturroz	1	1
2	F6	Alava, Puerto Herrera	1	2
3	F4	Burgos, Puerto Carrales	1	2
4	F2	Asturias, Tameza	2	2
5	F10	Asturias, Puerto Ventana	2	2
6	F11, F13, F14, I3–I6	León, Sierra de Ancares	2	2, 3
7	I2	Avila, Sierra de Gredos	3	6
8	F27, F29, F32, F33	Madrid, Puerto Guadarrama	3	6
9	F23, F25, F26, F31	Madrid, Puerto Navacerrada	3	6
10	F24, F28, F30	Madrid, Puerto de Cotos	3	6
11	F38	Cáceres, El Calvitero	3	6
12	F39	Madrid, Miraflores de la Sierra	3	5
13	F40, F41	Segovia, Pto. de Canencia	3	5
14	F42, F43	Madrid, Pto. de Navafría	3	5
15	F34, F35	León, Viaducto de Aralla	2	2
16	F7, F15–F22	Asturias, Arbás	2	2, 3
17	F3	León, Puerto Picones	2	2
18	F12	León, Rodrigatos de la Obispalía	3	5
		Portugal		
19	F36, F37	Sierra de Sintra	4	4

(a) Territories: 1. Cantabrian-Atlantic province; 2. Oro-Cantabrian province; 3. Carpetanic-Iberian-Leonese province; 4. Gaditanian-Onubian-Algarvian province.

(b) Bioclimatic belts: 1. Coline; 2. Montane; 3. Subalpine; 4. Thermomediterranean; 5. Supramediterranean; 6. Oromediterranean.

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 Glucose phosphate isomerase (GPI, EC 5.3.1.9) were assayed after horizontal 12% starch gel electrophoresis using the buffers and staining methods described by Figueiras *et al.* (1985) and Chenicek & Hart (1987).

Chromosome in situ *hybridization*

The chromosome preparations were pretreated prior to fluorescent *in situ* hybridization (FISH) with RNase and pepsin according to Wiegant *et al.* (1991). Chromosomes were denatured in 70% formamide, 2 × SSC for 2–3 min at 80°C and quickly immersed in cold 70% ethanol and ethanol dehydrated.

Total genomic DNA and clone pTa71 (Gerlach & Bedbrook, 1979) containing a 9 Kb EcoRI fragment of the wheat 18S-5.8S-26S rRNA gene repeat unit were used as probes. For experiments involving genomic *in situ* hybridization, DNA fragments of 100–200 bp length were obtained by autoclaving the total genomic DNA for 5 min and used as a block. The probe/block ratio was 1/100.

DNA probes were labelled by 'nick translation' with digoxigenin-11-dUTP (Boehringer Mannheim). Immediately prior to *in situ* hybridization the probes were mixed to a final concentration of 4 ng/µl in a solution of 50% formamide, 10% dextran sulphate, 0.5 M phosphate buffer and 2 × SSC. After hybridization for 16 h at 37°C,

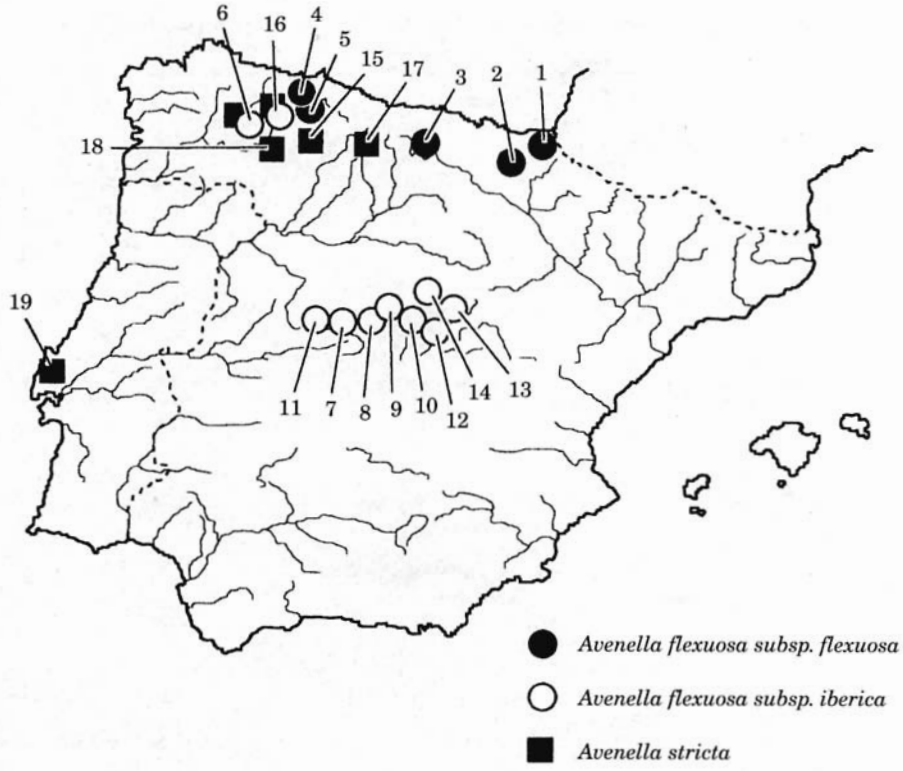

Figure 1. Geographical distribution of the localities where the samples were collected.

posthybridization washes were carried out at 37°C in 50% formamide, 2 × SSC, and 60°C 2 × SSC. The conditions used allow hybridization to occur between nucleotide sequences with at least 80% identity (Meinkoth & Wahl, 1984). The detection of signals involved three antibodies for the digoxigenin detection, the final one being conjugated to TRITC (tetramethylrhodamine β isothiocyanate) (red fluorescence). Chromosomes were counterstained in DAPI (diamidine phenylindole dihydrochloride). Observations were made and photographs taken using a Fluophot Nikon microscope and a 400 ASA colour slide film.

RESULTS

Plant habit

The plants analysed displayed two types of habit affecting plant size and leaf morphology (Fig. 2, Table 2). These differences in habit were maintained after four years under the same conditions of controlled cultivation in the greenhouse.

Micromorphology

All samples examined, except F2, F4, F5, F6 and F10, had trichomes on the abaxial surface of the base of the foliar laminas (Fig. 3, Table 2). No differences concerning type of foliar trichomes were obtained to characterize different groups.

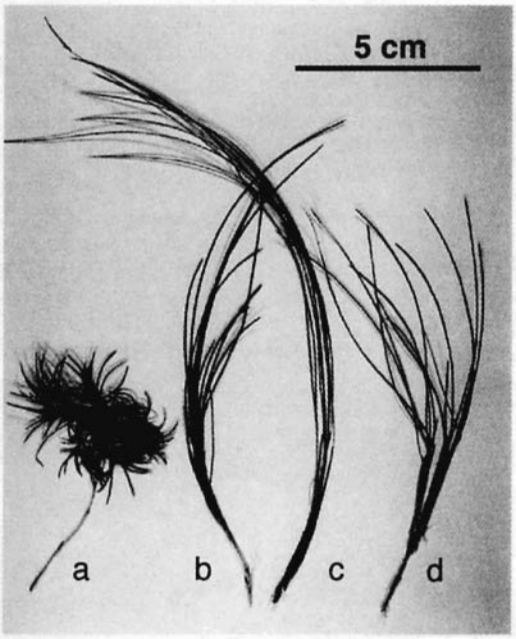

Figure 2. Vegetative shoots of different Avenella samples after four years of culture in the greenhouse; (a) sample F39, (b) sample F4, (c) sample F36, (d) sample F3.

TABLE 2. Habit, chromosome numbers, trichomes (+ = present, − = absent), and MDH patterns of the *Avenella* samples studied

Plant habit					
Plant size (cm)	Leaf morphology	Chromosome number	Trichomes	MDH type	Samples
10–30(–40)	Curved	$2n=28$	+	B	F11, F13, F14, F18–F21, F23–F33, F38–F43, I2–I4, I6
35–70	Straight	$2n=28$	−	A	F2, F4–F6, F10
35–70	Straight	$2n=56$	+	B	F3, F7, F12, F15–F17, F22, F34, F35, I5
35–70	Straight	$2n=56$	+	C	F36, F37

Karyotypes and C-banding pattern

The chromosome numbers of the different samples are summarized in Table 2.

The plants with $2n=4x=28$ chromosomes presented a karyotype comprising 14 pairs of submetacentric chromosomes (Fig. 4A), of which two pairs had secondary constrictions. Karyotypes comprising 28 pairs of submetacentric chromosomes (Fig. 4B) were observed in the samples with $2n=8x=56$ chromosomes. These karyotypes showed four pairs with secondary constrictions.

In both groups of plants (tetraploid and octoploid), all chromosomes were similar in size and most C-heterochromatin was forming telomeric C-bands. Some

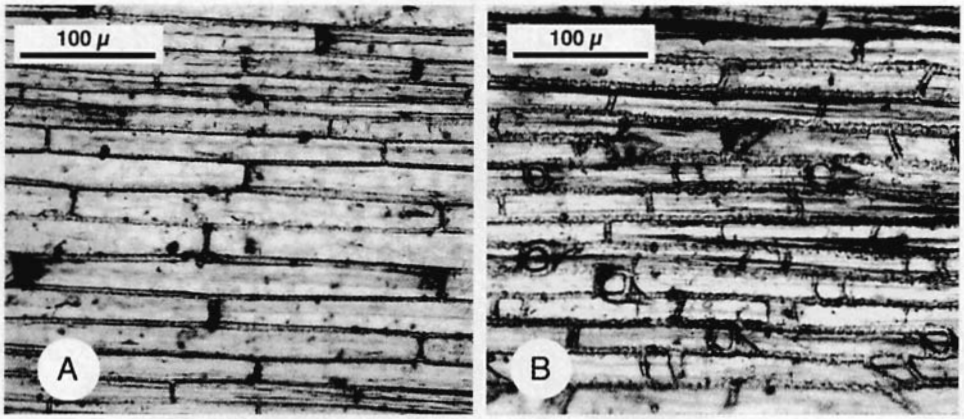

Figure 3. Abaxial surface of the base of foliar lamina. A, sample F10. B, sample F37. (× 200).

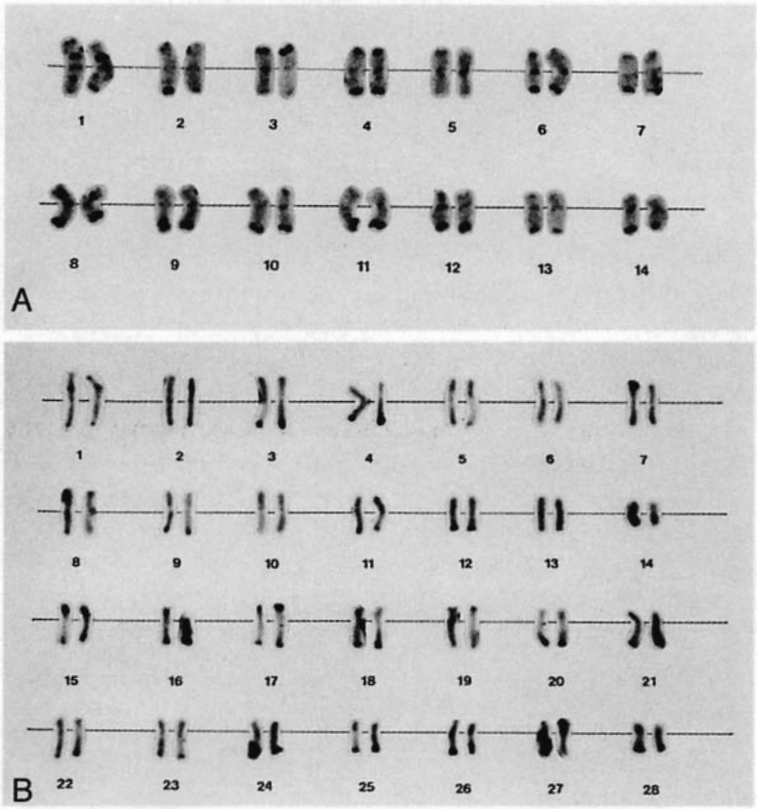

Figure 4. Examples of C-banded karyotypes. A, sample I3 ($2n = 4x = 28$). B, sample F7 ($2n = 8x = 56$).

chromosomes showed polymorphism for the amount of telomeric C-heterochromatin. Large interstitial bands were observed only in chromosomes carrying satellites, near the secondary constriction.

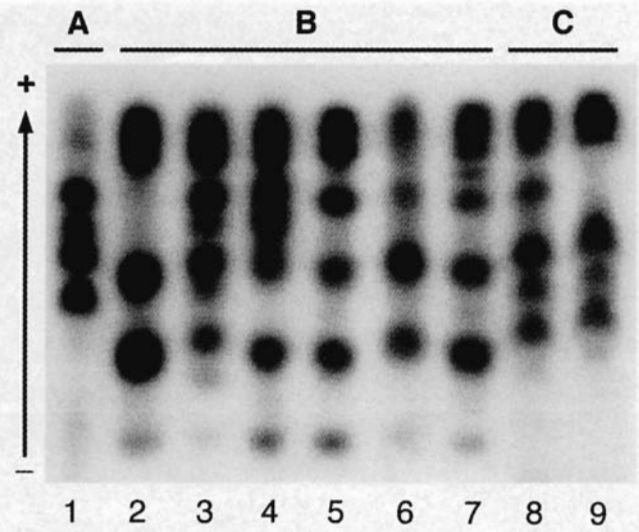

Figure 5. Electrophoretic pattern types (A, B and C) of MDH system. Lanes: 1, sample F10; 2, sample I6; 3, sample F18; 4, sample F27; 5, sample F33; 6, sample F15; 7, sample F34; 8, sample F36; 9, sample F37.

Isozyme assays

All samples showed a single band in the anodic region for the PGM isozyme system (data not shown). In the GPI isozyme system, polymorphisms showing two or three bands in the anodic region were observed between individuals belonging to a same population (data not shown). The relatively large variation in pattern found for the MDH isozyme system could be grouped into three different pattern types (Fig. 5, Table 2). Samples F2, F4, F5, F6, and F10 ($2n=28$) showed the pattern type A formed by several bands with intermediate mobility. Samples F36 and F37 (from Sierra de Sintra) showed bands with intermediate and high mobility (pattern type C). The remaining samples were characterized by the presence of a band with very low mobility (type B).

Chromosome in situ *hybridization*

With probe pTa71, four strong hybridization signals were detected on metaphase spreads of tetraploid ($2n=28$) plants (Fig. 6A). These signals show the sites of the major 18S-5.8S-26S rRNA gene clusters, located on two pairs of chromosomes associated with the secondary constrictions. An additional faint hybridization signal was observed in some tetraploid individuals. Eight strong signals localized in the secondary constrictions (Fig. 6B) were observed in all metaphases analysed from octoploid plants.

The genomic *in situ* hybridization was carried out on samples F3, F7 and F22 with $2n=56$ chromosomes. Digoxigenin-labelled total genomic DNA of tetraploid samples F2, F4, F20, F24, and F27 hybridized with the same intensity as all chromosomes of the octoploid plants (Fig. 6C). Similar results were obtained when the experiment was repeated, blocking with unlabelled genomic DNA from octoploid plants.

Figure 6C shows some chromosomes in which the telomeric region is blue,

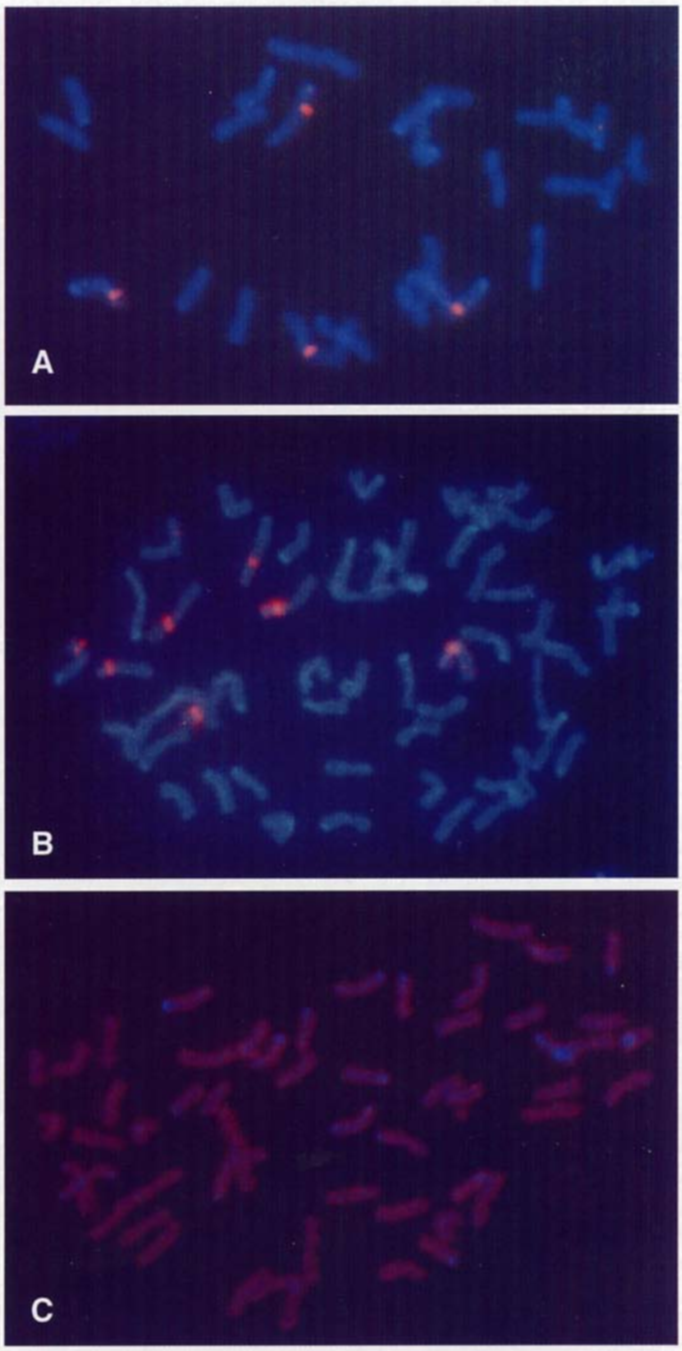

Figure 6. Chromosome *in situ* hybridization to root tip metaphase chromosomes. A, FISH of mitotic metaphase of a sample with $2n=28$ chromosomes hybridized with the rDNA probe pTa71 (red). B, FISH of mitotic metaphase of a sample with $2n=56$ chromosomes hybridized with the rDNA probe pTa71 (red). C, Genome *in situ* hybridization of mitotic metaphase of a sample with $2n=56$ chromosomes probed with Digoxigenin-labelled (red) total genomic DNA from a tetraploid ($2n=28$) sample. In all cases, the intensity of the DAPI counterstaining (blue) is higher in the telomeric region of some chromosomes due to the presence of C-heterochromatin in these regions (see Fig. 4).

presumably due to the DAPI staining coming through. A higher intensity of the DAPI staining can also be observed in the telomeric regions of some chromosomes in Figs 6A and 6B. This suggests that such staining differences are due to the presence of telomeric C-heterochromatin (see Fig. 4), instead of representing regions of genome divergence.

DISCUSSION

The occurrence of two levels of ploidy ($2n=4x=28$ and $2n=8x=56$) in the genus *Avenella* in the Iberian Peninsula was described by Albers (1980b). This author recorded the tetraploid level in *A. flexuosa* subsp. *flexuosa* from the north Iberian Peninsula and the octoploid level in *A. stricta* from south and west Iberian Peninsula. Our results show a wider geographical distribution of *Avenella* plants with those levels of ploidy (Fig. 1, Table 1).

No differences were observed in the C-banding pattern and in the heterochromatin amount between the samples analysed. These results are not in agreement with those obtained by Marschewski & Albers (pers. comm.), who found that endemic species of *Deschampsia* P. Beauv. have greater amounts of heterochromatin than species with wide areas of geographical distribution.

Only one pair of nucleolus organizing chromosomes, designated chromosome 1, was reported in mitotic cells of *Avenella flexuosa* ($2n=4x=28$) by Albers (1980a). After fluorescent *in situ* hybridization in tetraploid plants (Fig. 6A), four strong signals were detected in all metaphases at the secondary constrictions, indicating the presence of two main pairs of NOR bearing chromosomes. Occasionally one single minor site was detected on one additional chromosome. The signal strength of this site suggests a minor copy number of rRNA genes. Variability in copy number and size of the 18S-5.8S-26S rRNA genes is often observed in plants (Rogers & Bendich, 1987).

Total labelled genomic DNA does not allow the determination of the parental origin of chromosomes in the octoploid plants (Fig. 6C). All chromosomes showed the same intensity of hybridization when labelled genomic DNA from either one of the two types of tetraploid plants was used as probe. The same results were observed when unlabelled total genomic DNA from octoploid samples was used as competitive blocking. These data indicate that the different plants analysed are closely related.

According to karyotypes, morphological characters and isoenzymatic patterns, the plants analysed can be arranged into four groups (Table 2). The two groups including tetraploid plants ($2n=4x=28$) differ in plant habit and presence of trichomes, even after four years of culture under the same environmental conditions. They also differ in MDH pattern. Moreover, they occur in different territories and bioclimatic belts. From a systematic point of view and taking these data into account, the tetraploid samples with MDH pattern type A should be included in *Avenella flexuosa* (L.) Drejer subsp. *flexuosa*. The tetraploid samples with MDH pattern type B would correspond to *Avenella flexuosa* (L.) Drejer subsp. *iberica* (Rivas-Martínez, Izco & Costa) García-Suárez, Fernández-Carvajal & Fernández Prieto, comb. nov.; [basionym: *Deschampsia flexuosa* (L.) Trin. subsp. *iberica* Rivas-Martínez, Izco & Costa, *Trab. Dept. Bot. Fisiol. Veg. Madrid*, 3: 113 (1971)].

The difference between the two groups including octoploid plants ($2n=8x=56$)

is based only on MDH patterns. If they belong to the same species, this could be the result of a geographical isolation and fixation of different alleles in the two groups of populations analysed. These differences could also be explained under the assumption of a recurrent formation (multiple origin) of this polyploid level, as suggested by Soltis & Soltis (1995). Taxonomically, all octoploid plants belong to *Avenella stricta* (Hackel) Albers. Since the presence of this species in the Oro-Cantabrian and Carpetanic-Iberian-Leonese provinces has not been previously described, more studies are necessary in order to establish its precise systematic position.

ACKNOWLEDGEMENT

This work has been supported by grant DF/95-217-6 of the University of Oviedo.

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