An assessment of the origin of *Alopecurus geniculatus* L. based on 2C DNA amounts, NOR sites, RAPDs and cpDNA analysis

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ABSTRACT

Phylogenetic relationships were investigated between three diploid species of *Alopecurus: A. myosuroides* Huds. *A. aequalis* Sobol. and *A. bulbosus* Gouan. (2n = 14) and two tetraploid species: *A. pratensis* L. and *A. geniculatus* L. (2n = 28). Significant differences in 2C DNA amounts were found between the species: *A. aequalis* = 7·20 pg, *A. myosuroides* = 7·74 pg, *A. bulbosus* = 9·27 pg, *A. pratensis* = 13·25 pg and *A. geniculatus* = 16·17 pg when measured by flow cytometry using propidium iodide as the fluorochrome and *Hordeum vulgare* L. as an internal standard. Differences in 2C amount between diploid and tetraploid species were significant. The 2C DNA amount of *A. geniculatus* was more than twice that of either *A. aequalis* or *A. myosuroides* but less than twice that of *A. bulbosus*. Four nucleolar organiser region (NOR) sites, identified by fluorescent *in situ* hybridisation using a 9kb *Eco* R1 fragment of the 18S-5.8S-25S ribosomal DNA genes isolated from wheat (pTa71), were found in each of the diploid species. Six NOR sites were found in *A. geniculatus* and eight in *A. pratensis*. Cluster analysis of 135 fragments produced after RAPD analysis with twelve primers showed the highest genetic similarity was between *A. bulbosus* and *A. geniculatus*, whose chloroplast (cp)DNA $tRNA^{Leu1}$ intron sequences were shown to be identical. This suggests that *A. bulbosus* was the likely diploid progenitor of *A. geniculatus.* However, polyploidy was accompanied by loss of DNA and a reduction in the number of NOR sites.

KEYWORDS: chromosome number, flow cytometry, 2C nuclear DNA amounts, nucleolar organiser regions (NOR), *in situ* hybridization, pTa71, phylogeny, RAPDs, $tRNA^{Leu1}$ intron, cpDNA sequence.

INTRODUCTION

The genus *Alopecurus* (Poaceae) includes c. 50 species that are distributed in Europe, Asia Minor and North Africa. It is thought to have originated in the Mediterranean basin (Strelkova 1938) and has been introduced into North America and New Zealand. In Britain, *A. pratensis* L. is extensively cultivated as a forage crop and *A. myosuroides* Huds. is a major weed of arable crops.

Three diploid species (2n = 2x = 14), *A. myosuroides*, *A. aequalis* Sobol. and *A. bulbosus* Gouan, and two tetraploid species (2n = 4x = 28) *A. geniculatus* L. and *A. pratensis* are considered in this study as they formed part of earlier studies (Sieber & Murray 1979, 1980, 1981). The chromosomes of all five species are metacentric to sub-metacentric. Chromosomes can only be distinguished on the basis of size or shape in *A. myosuroides*, which contains a large pair of metacentric chromosomes (Sieber & Murray 1979).

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Alopecurus bulbosus is rare in Britain as it is at the northern edge of its distribution. It is restricted to a few populations on the margins of salt-marshes in southern England. Maude (1939), Darlington & Wylie (1956) and Fedorov (1969) reported only diploid plants of this species but Sieber & Murray (1980) found populations with diploid, triploid and tetraploid individuals (2n = 2x = 14, 2n = 3x = 21 and 2n = 4x = 28). It closely resembles *A. geniculatus* (Hubbard 1984) but may be distinguished by its erect habit and swelling of the basal nodes. Hybrids of *A. bulbosus* × *A. geniculatus (A.* × *plettkei* Mattf.) are triploid, sterile and morphologically intermediate between the two parental species. *Alopecurus aequalis* is an annual species found in sporadic populations at the margins of lakes and reservoirs in south-eastern England. *A. myosuroides* appears to be more distantly related to the other diploid species on morphology. Furthermore, both terminal and interstitial chiasmata were formed during meiosis in *A. myosuroides*, whereas only terminal chiasmata were found in *A. aequalis*, *A. bulbosus* and *A. geniculatus* (Sieber & Murray 1979). Previous studies have suggested that *A. geniculatus* is an autotetraploid sharing common ancestry with *A. aequalis* and *A. bulbosus*, as does one of the genomes of the allotetraploid *A. pratensis* (Johnsson 1941; Sieber & Murray 1979, 1980, 1981; Murray *et al*. 1984). *A. pratensis* and *A. geniculatus* hybridise in natural populations and B chromosomes have been reported in *A. pratensis* (Sieber & Murray 1981).

Nuclear DNA amounts have been measured by Feulgen microdensitometry (Sieber & Murray 1979; Olszewska & Osiecka 1982; Bennett & Smith 1991; Bennett & Leitch 1997) and are summarised in Table 1. With the exception of Sieber & Murray (1979), the studies are limited as the chromosome number of the plants studied was not recorded and the DNA amounts cannot be compared precisely with those of the present investigation.

Flow cytometry enables DNA amounts to be determined rapidly from leaf tissue. The choice of a suitable fluorochrome is important. The fluorochrome 4´, 6-diamidino-2-phenylindole (DAPI) results in lower coefficients of variation of peaks of DNA content histograms than other commonly used fluorochromes including propidium iodide (PI) but has a preference for AT bases (Doležel *et al.* 1992). Whilst PI shows no base preference and is therefore more suitable than DAPI for the determination of absolute DNA amounts. *Hordeum vulgare* cv Sultan (2C = 11·12 pg) (Bennett & Leitch 1995) was used as an internal standard in this investigation because it is one of the standards recommended by Bennett & Leitch (1997) and its DNA amount is similar to that of *Alopecurus* species.

TABLE 1. ESTIMATES OF 2C NUCLEAR DNA AMOUNTS (MEAN ± SD) OF FIVE *ALOPECURUS* SPECIES ESTIMATED BY FLOW CYTOMETRY, USING PROPIDIUM IODIDE, (THIS INVESTIGATION) AND FEULGEN MICRODENSITOMETRY IN PREVIOUS STUDIES

	Present study (Propidium Iodide)			Previous studies (Feulgen)		
Species	Chromosome no. (2n)	Mean 2C (pg DNA ± SD)	Standard	Chromosome no. (2n)	Mean 2C (pg DNA ± SD)	Standard
A. aequalis	14	7·20 ± 0·11	A	14	8·6 ± 0·35[1]	*C*
A. myosuroides	14	7·74 ± 0·16	A	14	9·4 ± 0·31[1]	*C*
				-	8·61[4]	*A*
A. bulbosus	14	9·27 ± 0·15	A	-	10·4 ± 0·28[1]	*C*
A. pratensis	28	13·25 ± 0·14	A	28	15·1 ± 1·38[1]	*C*
				-	13·62[2]	*B*
A. geniculatus	28	16·17 ± 0·14	A	28	18·5 ± 0·12[1]	*B*
				-	14·93[3]	*D*

(1. Sieber & Murray 1979; 2. Olszewska & Osiecka 1982; 3. Bennett & Smith 1991; 4. Bennett & Leitch 1997). The standards used were A) *H. vulgare* cv Sultan (2C = 11·12 pg), B) *Allium cepa* cv Ailsa Craig (2C = 33·5pg), C) *Allium cepa* cv Suttons A1 (2C = 33·5pg), D) *Senecio vulgaris* (2C = 6·33pg).

In recent years fluorescent *in situ* hybridisation (FISH) techniques have been developed (Heslop-Harrison *et al.* 1991; Kenton *et al.* 1997; Leitch *et al.* 1997). The clone pTa71, containing the 9Kb Eco R1 fragment of wheat ribosomal DNA (rDNA) (Gerlach & Bedbrook 1979), has been used to detect the nucleolar organiser regions (NOR sites) of highly repeated and tandemly arrayed 18S, 5.8S and 25S rDNA sequences (Flavell 1986). This method has been used to clarify relationships and evolution in a number of polyploid species, including members of the Triticeae (Mukai *et al*. 1991; Carbrera *et al.* 1995; Jiang & Gill 1994), *Festuca* (Thomas *et al.* 1997), *Lathyrus* (Murray *et al.* 1992a), and *Saccharum* (Jenkin *et al*. 1995).

Traditional systematics relies on morphological characteristics and meiotic pairing to differentiate between taxa. DNA-based molecular techniques have made considerably more information available. However, limitations in the type and appropriate use of data generated exist (Karp *et al*. 1996). Random amplified polymorphic DNA (RAPD) has been used to evaluate the genetic structure of populations, species and genera in a wide range of plants: *Brachypodium* (Catalan *et al*. 1995), *Festuca/Lolium* (Stammers *et al*. 1995), *Brassica* (Demeke *et al*. 1992), *Rosa* (Millan *et al*. 1996), and *Rubus* (Graham & McNicol 1995). However, phylogenetic trees generated from RAPD data should be treated with caution, as there are a number of problems with using RAPD generated patterns for phylogenetic analysis that introduces noise into RAPD data sets (Reiseberg 1996; Gillies & Abbott 1998) and it has not been possible to produce phylogenies for *Leucaena* (Harris 1995) and *Styloanthes* (Gillies & Abbott 1998) using RAPD data. Nonetheless, conserved bands do occur within closely related species that are useful for phylogenetic analysis (Harris 1995; Stammers *et al. 19*95).

As chloroplast DNA (cpDNA) sequences have a conservative rate of nucleotide substitution and a uniparental (maternal in most plant species) mode of transmission they have been widely used to generate phylogenies within and between genera (Wolfe *et al*. 1987, 1989; Downie & Palmer 1992). Comparison of the rates of mutation in *rbcL* and two non-coding regions of cpDNA, the *trnL* (UAA intron) and the intergenic spacer between the *trnL* (UAA) 3' exon and the *trnF* (GAA) gene, have been made in several genera. These non-coding regions evolve faster and show greater resolution at the generic and intrageneric level (Taberlet *et al.* 1991; Gielly & Taberlet 1994), and phylogenies based on *trnL* intron sequences have been generated for several genera (Taberlet *et al*. 1991; Ferris *et al.* 1993; Gielly & Taberlet 1994; Fennell *et al.* 1998). The *trnL* gene of chloroplasts and eubacteria contain a group I intron that is conserved in primary sequence, secondary structure and its position within the UAA anticodon of the gene. Whilst some regions are highly conserved (Kuhsel *et* al. 1990; Ferris *et* al. 1993) other regions, in particular the region between the sequences of the stem P8, are variable and have been found to contain insertion/deletion mutations and microsatellite variation in addition to simple base substitutions between species within the genus *Spartina* (Ferris *et al*. 1997; Ferris, unpubl.). The conserved regions greatly aid alignment of sequences while the variety of mutations provides useful phylogenetic data. Thus the cpDNA *trnL* intron from the five species of *Alopecurus* was sequenced to provide further phylogenetic comparisons.

The aim of this study was to use the techniques of molecular systematics to study the relationships between the five *Alopecurus* species, in particular to identify the diploid progenitor of the tetraploid species. The DNA amounts of the five species of *Alopecurus* were estimated both to resolve apparent inconsistencies between previously published DNA amounts and to examine the evidence of the possible origins of the tetraploid species. NOR sites were identified using FISH to provide further information on the possible diploid progenitor of the tetraploid species.

MATERIALS AND METHODS

Plants investigated

Plants of each species of *Alopecurus* were taken from natural populations (Table 2) and maintained in pots in an experimental garden.

DNA MEASUREMENTS BY FLOW CYTOMETRY

The 2C DNA content of isolated nuclei obtained from leaves was determined by flow cytometry using *H. vulgare* cv. Sultan (2C = 11·12 pg) as the internal calibration standard and a Partec CA-III flow Cytometer (Partec GmbH, Munster, Germany). The method used to measure DNA amount

TABLE 2. LOCATION OF PLANT COLLECTIONS AND TYPE OF ASSAY PERFORMED ON INDIVIDUAL SPECIMENS AND ASSAY

Species	Site	Grid Reference	Assay
A. aequalis	Boddington Reservoir, Warks.	SP498525	2
A. aequalis	Ruislip Lido, Bucks.	TQ087895	1 + 2
A. aequalis	Heronry Pond, Wanstead Flats, Essex	TQ415871	3
A. bulbosus	Seasalter, Sussex	SY463906	2
A. bulbosus	Bridport, Dorset	SY463906	1 + 2 + 3
A. myosuroides	Epping Forest, Essex	TQ425995	2
A. myosuroides	The Abbey, Tilty, Essex	TL600268	1 + 2
A. myosuroides	Moletrap Pub, Tawney Common, Essex	TL501013	3
A. pratensis	Wanstead Common, Essex	TQ400865	2
A. pratensis	Leamington Spa, Warks.	SP325657	1 + 2
A. pratensis	Plymouth, Devon	SX515626	3
A. geniculatus	Leamington Spa, Warks.	SP325657	1 + 2 + 3
A. geniculatus	St Petersburg, Russia	-	2

1. flow cytometry, 2. RAPD analysis, 3. sequencing *trnL* cpDNA.

using PI as the fluorochrome was that of Doležel *et al.* (1989). Approximately 50 mm^2 of young leaves of the *Alopecurus* under investigation together with a similar area of leaf material of *H. vulgare* cv. Sultan were chopped together in 400µl nuclei isolation buffer (lysis buffer, LB01) (Doležel *et al. 19*89), consisting of 15 mM Tris, 2 mM NaEDTA, 80 mM KCl, 20 mM NaCl, 0·5 mM spermine, 15 mM ß-mercaptoethanol, 1 ml l^{-1}Triton X-100, pH 7·5. All reagents were obtained from Sigma Ltd. unless otherwise stated. The lysate was made up to 2 ml with buffer (LB01) and filtered through nylon gauze (pore diameter 40 µm). 50µg/ml^{-1} ribonuclease A and 50µg/ml^{-1} PI were added and the suspension incubated in the dark for one hour on ice. The samples were filtered through nylon gauze (pore diameter 20 µm) and fluorescence was measured with a 40 × 0·80 quartz objective and an Argon laser light source (488 nm wavelength) used with a TK420 dichromic mirror and an OR610 barrier filter. The data were analysed on DPAC software (Partec GmbH, Münster, Germany). The linearity of measurements of fluorescence intensity obtained was checked by the method of Bagwell *et al.* (1989). However, no correction was required to compensate for non-linear amplification by the flow cytometer. Estimates of the ratio of fluorescence intensities of *Alopecurus* to *Hordeum* (p1/p2) were based on the mean of a minimum of three samples per plant, each with a minimum of 10,000 nuclei giving peaks with a coefficient of variation of less than 5%. The product of this ratio and the DNA amount of *H. vulgare* (11·12pg) was used to provide an estimate of DNA amount for each *Alopecurus* species, measurements were made from at least three individual plants.

Estimates of the 2C ratio of DNA for the five species were also made using 4´, 6-diamidino-2-phenylindole (DAPI) using the method of Yokoya *et al.* (2000).

CHROMOSOME COUNTS

The tips of actively growing roots or shoots were pretreated in a saturated solution of α-bromonaphthalene at 4ºC for 20 – 22h. They were then fixed in 3:1 absolute ethanol: glacial acetic acid (v/v). Following hydrolysis for 10 min in 5N HCl at 20ºC, the meristems were dissected out and squashed between microscope slide and coverslip in 0·1% aceto-orcein. For each species, a minimum of five metaphases were counted for each of the three plants used for flow cytometry.

NOR SITES

The tips of actively growing roots were pretreated as above. The root was placed in a drop of 0·01 M sodium citrate buffer on a microscope slide and the meristem excised with tungsten needles. Excess buffer was removed and a drop of 0·01M sodium citrate buffer containing 6% cellulase and

2% pectolyase (Kikkoman Ltd.) added. Slides were then incubated in a humid chamber at 37°C for 15–20 minutes, after which the enzyme mixture was removed. Excess epidermal tissue was removed, the root tip was macerated in 45% acetic acid and then squashed between slide and coverslip. Coverslips were removed from frozen preparations. The slides were then air dried, and stored at -70°C in a sealed bag with silica gel.

NOR sites were identified using a 9kb *Eco* RI fragment including the 18S-5.8S-25S ribosomal DNA genes isolated from wheat (Gerlach & Bedbrook 1979) contained in plasmids (pTa71). The method used was essentially that of Heslop-Harrison *et al.* (1991). Root tips were prepared as above. Excess cytoplasm was removed from air-dried squash preparations by treatment with 10µg/ml^{-1} pepsin at 37°C in a humid chamber (Leitch *et al. 19*94). The slides were dehydrated through a series of graded alcohols and stored overnight in a vacuum desiccator at 4°C to stabilise the chromosome morphology (Leitch *et al. 19*94; Leitch *et al.* 1997). Prior to hybridisation the slides were placed in 3:1 absolute ethanol: glacial acetic acid (v/v) for 30 minutes and in 96% ethanol twice for 10 minutes before air-drying (Kenton *et al.* 1997). Hybridisation of the probe to the chromosome preparations followed the protocol of Heslop-Harrison *et al.* (1991) using an Omnigene *in situ* block (Hybaid Ltd). The hybridisation mix (per 40 µl of mix: 20µl 100% formamide, 8µl 50%(w/v) dextran sulphate, 4µl 20xSSC, 2µl of probe DNA (pTa71) (100ng/µl), 2µl of sonicated herring sperm DNA, 0·5µl 10%(w/v) SDS and 3·5µl of water) was heated to 80°C for 10 min, cooled on ice for 5 min before placing 40 µl of the hybridisation mix on a single slide which was covered with a plastic coverslip and placed on the Omnigene *in situ* block. Chromosomes were denatured at 72°C and washed. Post-hybridisation washes, after overnight incubation on the PCR block at 37°C, followed Heslop-Harrison *et al.* (1991). The slides were given two stringency washes in 20% (v/v) formamide in 0·1 × SSC for 5 min at 42°C in a shaking water-bath, followed by three further 3 min washes in 2 × SSC at 42°C. Probe bound to the chromosomes was detected using fluorescein isothiocyanate (FITC) labelled anti-digoxigenin (Boehringer-Mannheim). The chromosomes were counter-stained using PI (2µg/ml) for 10 min in the dark, washed in phosphate buffered saline and mounted in Vectarshield (Vector Laboratories Ltd.) containing 2 µg/ml DAPI. Slides were viewed with a Nikon Optiphot fluorescent microscope with a microflex UFX-11 attachment using the G filter (546nm). The negatives were digitised at 80 dpmm (Boots plc), imported into Adobe Photoshop and converted to greyscale.

DNA isolation

Genomic DNA was extracted from fresh leaves in CTAB buffer using a modification of the method of Weising *et al.* (1995). Excess protein was removed by digestion with protease, followed by washing, to remove carbohydrates (Jobes *et al.*1995; Sul & Korban 1996). All reagents were obtained from Sigma Ltd unless otherwise stated. Briefly, 1g of fresh leaves harvested from greenhouse grown plants was quickly frozen in liquid nitrogen and ground in a pre-chilled pestle and mortar. The powder was mixed with 7 ml 1:1 3% CTAB buffer: 8M LiCl (v/v) and incubated for 1 hr at 60°C (Sul and Korban 1996). The DNA was washed twice with an equal volume of 24:1 (v/v) chloroform/isoamyl alcohol and mixed thoroughly to form an emulsion, which was centrifuged at 4,400 rpm for 10 min and the supernatant transferred to new tubes. The chloroform/ isoamyl step was repeated once. The DNA was precipitated with 2/3 volumes of ice-cold isopropanol held at -20°C for a minimum of 30 min before centrifuging at 4,400 rpm for 5 min and resuspending in TE buffer with 0·5% SDS (Sambrook *et al*. 1989). Excess proteins were removed by treatment with 1mg/ml of protease (from *Streptomyces griseus*) at 37°C for 2hrs. The DNA was precipitated with isopropanol as above and centrifuged at 13,000 rpm for 10 min. The pellet was dissolved in water. 0·5 volume 5M NaCl (v/v) and then two volumes of 95% ethanol were added to precipitate the DNA (Jobes *et al.* 1995). The pellet was resuspended in 500 µl H_2O. The DNA was then washed twice with 25:24:1 (v/v) phenol/chloroform/isoamyl. RNA was removed by treatment with 150 µg/ml of RNAse A for 30 min at 37°C. The DNA was precipitated in 1/10 volumes of 4M LiCl followed by 3 volumes of ice-cold absolute ethanol (v/v), centrifuged at 13 000 rpm for 15 minutes and dissolved in 50 µl of TE buffer. The DNA was stored at 4ºC for up to one month or -20ºC for up to 6 months. DNA quality and quantity was determined by gel electrophoresis. A 5 µl sample of the DNA was mixed with 3 ml of loading buffer. The electrophoresis was performed on 0·8% agarose gel in TAE buffer (Sambrook *et al.* 1989) containing 0·5 ng of ethidium bromide using a Hybaid Electro4 with an LKB Bromma 2179 power supply. DNA concentration was estimated by visual assessment of band intensities, compared to a lambda *Hind*III ladder (Promega Ltd).

RAPD analysis

DNA from a minimum of two plants per species, each from a different population, (Table 2) was used for RAPD analysis using a modification of Williams *et al.* (1990). Optimised reaction conditions were carried out on approximately 50 ng genomic DNA in 25 µl total volume containing 2·5 µl of 10x *Biotaq* buffer (Bioline Ltd), 2·5µl 25 mM $MgCl_2$, 2·0 µl of 10 mM dNTP's (dGTP, dCTP, dATP, dTTP) (Bioline Ltd), 0·1µl of 5Uμl^{-1} *Biotaq* polymerase (Bioline Ltd.) and 10 µl of primer (10mM concentration, Bioline Ltd). Each reaction mix was overlaid with PCR-grade paraffin oil. The PCR profile was as follows: 1 denaturation cycle of 95°C for 2 min, then 2 cycles of 95°C for 30 sec, 37°C for 1 min, 72°C for 2min, then 2 cycles of 95°C for 30 sec, 35°C for 1min, 72°C for 2 min, then 41 cycles of 94°C for 30 sec, 35°C for 1 min, 72°C for 2 min and terminated with a final extension of 72°C for 10 min.

Amplified DNA fragments were separated by 1·6% agarose gel electrophoresis using TAE buffer (Sambrook *et al. 19*89). A negative control was added in each run to test for contamination. A 100 bp ladder (Promega Ltd.) was included on all gels to provide a standard DNA sample. Gels were stained with 0·5 µg ml^{-1} ethidium bromide and fragment patterns were photographed under UV light using Polaroid 667 film. The presence of bands was verified by eye, however the Polaroid photographs were used for the analysis and to determine the molecular weight of each visible band.

Twenty primers were evaluated for their suitability and twelve 10-base primers (Bioline Ltd) were selected on the basis of the reproducibility and intensity of polymorphic bands (Table 3). The selection of each primer was based, after several trials, on its ability to amplify the DNA from all five species and that each primer gave reproducible bands on more than one occasion from DNA from the same plant. Bands of identical size amplified with the same primer were considered to originate from the same marker. Markers that were considered unreliable were excluded and only data from clear bands were scored.

Bands were scored as present (1) or absent (0), and a matrix constructed such that the columns corresponded to the individual plants analysed and the rows to the presence or absence of bands. Pairwise genetic similarities were calculated using Jaccard's coefficient of asymmetric similarity (F), where $F=M_{xy}/(M_t-M_{xyo})$, where M_{xy} represents the number of bands shared between both individual plants, M_t the total number of bands in the survey and M_{xyo} the number of bands that were not present in either of the individuals (Jaccard 1908; Gillies & Abbott 1998; Virk *et al.* 1995) in RAPDistance v1.01 (Armstrong *et al.* 1994). The unweighted pair group method with arithmetic average (UPGMA) similarity and cluster analysis in RAPDistance and PHYLIP v.3.5 (Felsentein 1993) were used to construct a phenogram showing genetic similarity between the five species.

Sequencing of the cp DNA $tRNA^{Leu1}$ (trnL) intron and parsimony analysis

DNA samples were extracted with a Qiagen extraction kit (Qiagen Ltd). The intron was amplified following the protocol of Taberlet *et al.* (1991) with the following modifications. In a sterile 0·5 ml eppendorf tube 4·0 µl of 10mM dNTPs were mixed with 5 µl of 10 × *Biotaq* buffer (supplied with enzyme by Bioline Ltd), 2·5 µl of 50mM $MgCl_2$, 0·4 µl of *Biotaq* polymerase, 1·0 µl of each 10mM primer (Table 4, Bioline Ltd), 10 µl of the diluted DNA sample (30–50 ng of DNA sample per tube), and 28·1µl of distilled water. The reaction mixture in each tube was overlaid with a drop of sterile mineral oil. For amplification the tubes were placed in a Perkin Elmer 9600 thermal cycler. The PCR programme was 5 minutes at 94°C, 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and two minutes at 72°C. A final ten minutes at 72°C ensured full extension of all amplified fragments. The amplification product was then purified using a Qiagen PCR Clean-Up Kit (Qiagen Ltd.). Direct PCR sequencing of the purified PCR products was carried out using the DyeDeoxy Terminator sequencing Kit (Applied Biosystems). The $tRNA^{leu1}$ intron was labelled with the supplied fluorescent dye mixture using a Perkin Elmer 9600 thermal cycler set to 25 cycles of the following sequence: 93°C for 30 sec, 50°C for 15 sec and 60°C for four min.

The DNA sequence was determined by automated acrylamide gel electrophoresis (Model 373A, Applied Biosystems). The sequences obtained from the five *Alopecurus* species were analysed with Sequence Navigator v.1.01 (ABI prism transbiosystem Inc.) and then transferred to Editseq v.3.75 (DNAstar). The $tRNA^{Leu1}$ intron sequences of eight species from the Poaceae from the EMBL database were used as an outlying group. The choice of species was made to represent a

TABLE 3. TYPE OF RAPD PRIMER, PRIMER SEQUENCE AND BAND DATA FOR EACH OF THE TWELVE SELECTED PRIMERS USED ON THE FIVE SPECIES OF *ALOPECURUS* PRODUCING A TOTAL OF 135 FRAGMENTS

Primer	Sequence 5'–3'	Total Fragments	Polymorphic Fragments	Monomorphic Fragments	Species Specific Fragments	Fragments Specific to *A. bulbosus* and *A. geniculatus*	Fragments Specific to *A. aequalis* and *A. geniculatus*
G-70-11	GTCTCGTCGG	7	7	0	*A. pratensis* *A. bulbosus*		
G-70-12	GGCCTACTCG	10	9	1	*A. myosuroides* *A. bulbosus* (2) *A. aequalis*		
G-70-16	CAGGGGCATC	8	8	0	*A. myosuroides* (4) *A. aequalis*		
G-70-1	GAGACCTCCG	15	15	0	*A. myosuroides* *A. geniculatus* *A. pratensis* (3) *A. bulbosus*	900bp 976bp	560bp
G-70-18	GGCCTTCAGG	18	18	0	*A. myosuroides* *A. aequalis* *A. pratensis* (2)	1050bp	1300bp
G-17-19	GCTCTCACCG	10	9	1	*A. myosuroides* *A. pratensis*	680bp	440bp 230bp
G-80-21	ACGCGCCAGG	9	9	0	*A. myosuroides*	550bp	
G-80-23	GGCCCCATCG	16	16	0	*A. myosuroides* *A. aequalis* *A. pratensis*	2500bp	
G-80-24	CGCGAGGTGC	8	7	1	*A. pratensis*		
G-80-26	CCCGAGTGCC	9	9	0	*A. myosuroides* *A. pratensis*	400bp	
G-80-28	CGCACCGCAC	12	12	0	*A. myosuroides* (4) *A. pratensis* *A. bulbosus*	930bp	1350bp
G-80-29	ACGGCGGCTC	13	12	1	*A. pratensis* *A. bulbosus* *A. geniculatus*		950bp
Total		135	131	4	42	8	6

TABLE 4. NUCLEOTIDE SEQUENCE AND ANNEALING TEMPERATURE OF PRIMER PAIR USED FOR PCR AMPLIFICATION OF $tRNA^{LEU1}$ cpDNA

Primer pair	Nucleotide sequences	Annealing Temp
trn L (UAA) 5'exon	5'CGAAATCGGTAGACGCTACG3'	55°C
trn L (UAA) 3'exon	5'GGGGATAGAGGGACTTGAA3	55°C

taxonomically closely related taxon, *Puccinellia distans*, to act as a sister to the ingroup, and also several more distantly related genera to adequately root the tree and indicate the general taxonomic position of the genus *Alopecurus* within the Poaceae. The EMBL reference numbers of sequences used to construct the tree are given in Table 5. Sequences for all taxa were aligned using Megalign v.1.05 (DNAstar Inc.) and the resulting matrix corrected manually to provide the optimum sequence alignment. The sequence data for all species were then analysed using PAUP v.4.0 (Swofford, 2002) to produce phylogenetic trees. A matrix scoring deletions and insertions was first added to the sequence data with the alignment gaps scored as missing data. All characters were weighted equally and unordered. A parsimony analysis was run using Branch and Bound with the COLLAPSE and MulTrees options on. Computation was via stepwise with the addition sequence set to furthest. As a test for levels of homoplasy within the data set the tree scores CI (consistency index), RI (retention index) and RC (rescaled consisteny index) were calculated. A bootstrap analysis was run using the same parameters as above with 1000 replicates.

TABLE 5. $tRNA^{LEU1}$ INTRON SEQUENCES USED FOR PHYLOGENETIC ANALYSIS

Species	EMBL accession number	Reference
A. myosuroides	AJ271688	This study
A. aequalis	AJ271686	This study
A. bulbosus	AJ271685	This study
A. geniculatus	AJ271689	This study
A. pratensis	AJ271687	This study
Oryza sativa	X15901	Hiratsuka *et al*. 1989
Hordeum vulgare	X745741	Fangan *et al.* 1994
H. vulgare 2	X757051	Gielly and Taberlet 1994
Puccinellia distans	X75706	Gielly and Taberlet 1994
Spartina alterniflora	Z69914	Ferris *et al*. 1997
S. maritima	Z69911	Ferris *et al*. 1997
Triticum aestivum	X75709	Gielly and Taberlet 1994
Zea mays	X75709	Maier *et al.* 1995

RESULTS

DNA amounts of leaf tissue measured by flow cytometry.

The mean 2C DNA values obtained in this study are shown in Table 1 together with estimates of DNA value from previous studies using Feulgen microdensitometry. The 2C DNA amounts obtained for the five species were significantly different and it was possible to discriminate between species using fluorescence intensities of PI-stained nuclei. There were significant differences between the mean 2C DNA values for species between and within both the diploid and the tetraploid species, (two-way ANOVA: $F = 1{\cdot}57$, $P = 0{\cdot}175$). There was a correlation between measurements made using PI and DAPI as the fluorochrome ($R^2 = 0{\cdot}9858$, $r = (p > 0{\cdot}05)$, $y = 1{\cdot}0658x$). Figure 1 shows the 2C DNA amounts of *Alopecurus* spp. estimated by flow cytometry using PI as the fluorochrome and previously published values obtained by Feulgen microdensitometry. The values obtained by Feulgen microdensitometry were, on average, 15% higher than those obtained by flow cytometry using PI as the fluorochrome. Of the diploid species,

A. bulbosus has the largest 2C DNA content of 9·27pg compared to those of *A. aequalis* (2C = 7·20 pg) and *A. myosuroides* (2C = 7·74pg). The DNA content of the tetraploid species *A. geniculatus* (2C = 16·17pg) is 87% of that of the doubled DNA content of *A. bulbosus*, but is 98% of the sum of the 2C DNA contents of *A. aequalis* and *A. bulbosus*.

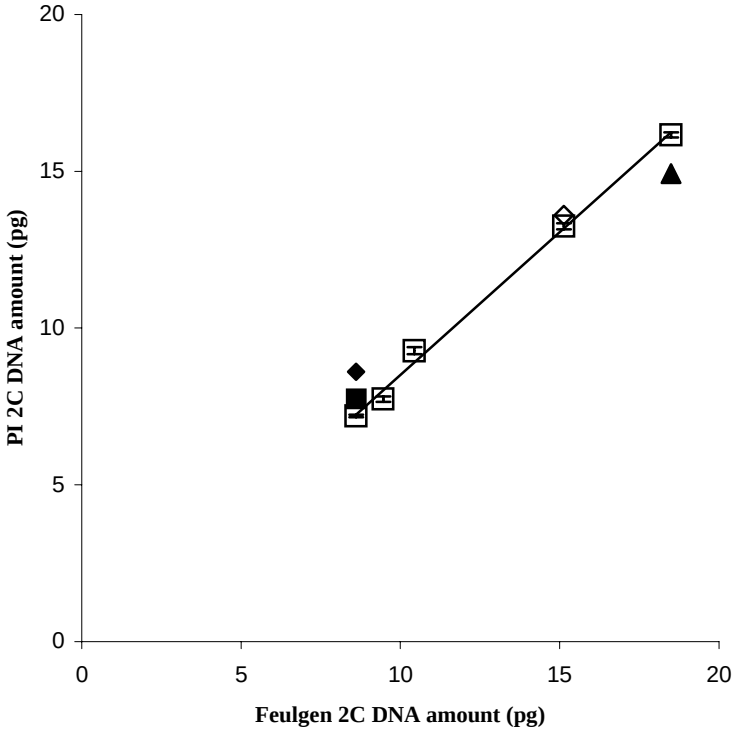

FIGURE 1. The mean 2C values of *Alopecurus* spp. obtained by flow cytometry using PI (□) as fluorochrome with *H. vulgare* cv Sultan as standard compared with values obtained by Feulgen microdensitometry using *A. cepa* cv Suttons A1 (Sieber & Murray 1979). Error bars indicate ± SE. The correlation between PI and Feulgen microdensitometry was R^2 = 0·9964, y = 0·9102x–0·584, r = (p>0·05). 2C values obtained from Feulgen microdensitometry for *A. myosuroides* (◆) (Bennett & Leitch 1997), *A. pratensis* (◇) (Olszewska & Osiecka 1982) and *A. geniculatus* (▲) (Bennett & Smith 1991).

Chromosome numbers and NOR-sites

The chromosome numbers of the populations analysed were found to be 2n = 14 for *A. aequalis*, *A. bulbosus* and *A. myosuroides* and 2n = 28 for *A. pratensis* and *A. geniculatus.* The chromosomes for all the species were metacentric or submetacentic. NOR sites were found at similar sub-terminal positions in all species. All the diploid species had 4 NOR sites whilst 6 NOR sites were found in *A. geniculatus* and 8 in *A. pratensis* (Fig. 2).

RAPDs

A total of 135 bands were scored, with an average of 11–25 bands per primer. 97% of bands were polymorphic, of which 31% were unique to only one species and 3% monomorphic. Fig. 3 shows the RAPD products of *Alopecurus* species generated from primer G-80-23. Table 3 gives the RAPD band data for each of the 12 primers and the 135 bands produced. Each primer had between 7 and 18 bands, which ranged in size from 230–2,500 bp, although the majority of bands occurred between 400–1500 bp. For each primer the species that have species-specific fragments are given, for those that have multiple specific fragments the number of fragments is indicated in brackets. Those fragments that are shared by only *A. bulbosus* and *A. geniculatus* and those that are only

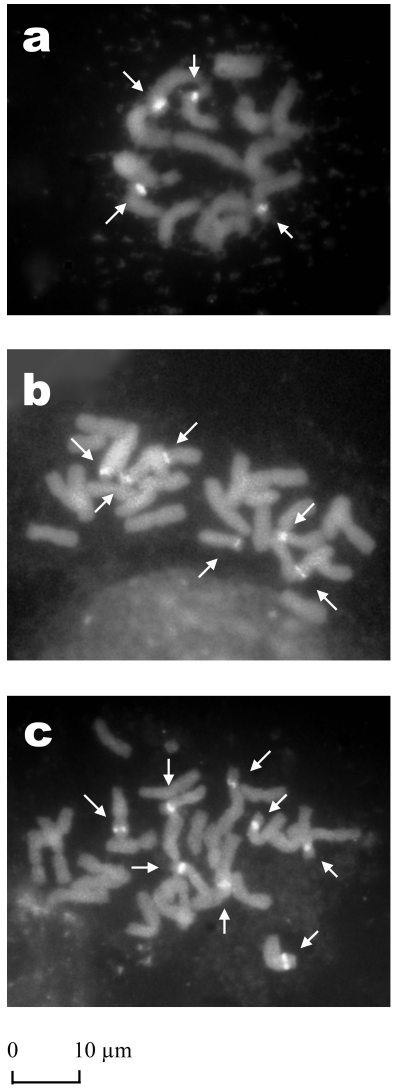

FIGURE 2. NOR-banded metaphases of a) *A. aequalis*, b) *A. geniculatus* and c) *A. pratensis* following FISH using plasmid pTa71 identified by FITC labelled antidigoxigenin and counterstained with PI; the NOR sites appear as bright signals on the chromosomes (arrows).

shared by *A. aequalis* and *A. geniculatus* are also listed as fragment sizes. However, it is likely that the number of species specific fragments and the bands shared between either only *A. geniculatus* and *A. bulbosus* and only *A. geniculatus* and *A. aequalis* would be reduced if a larger sample size had been used. Although intraspecfic variation occurred for all the species, the sample size used in this study limits any conclusions on this point. UPGMA cluster analysis of similarity showed that the highest degree of similarity occurred between *A. geniculatus* and *A. bulbosus* whilst the greatest differences occurred between *A. geniculatus* and *A. myosuroides* (Fig. 4).

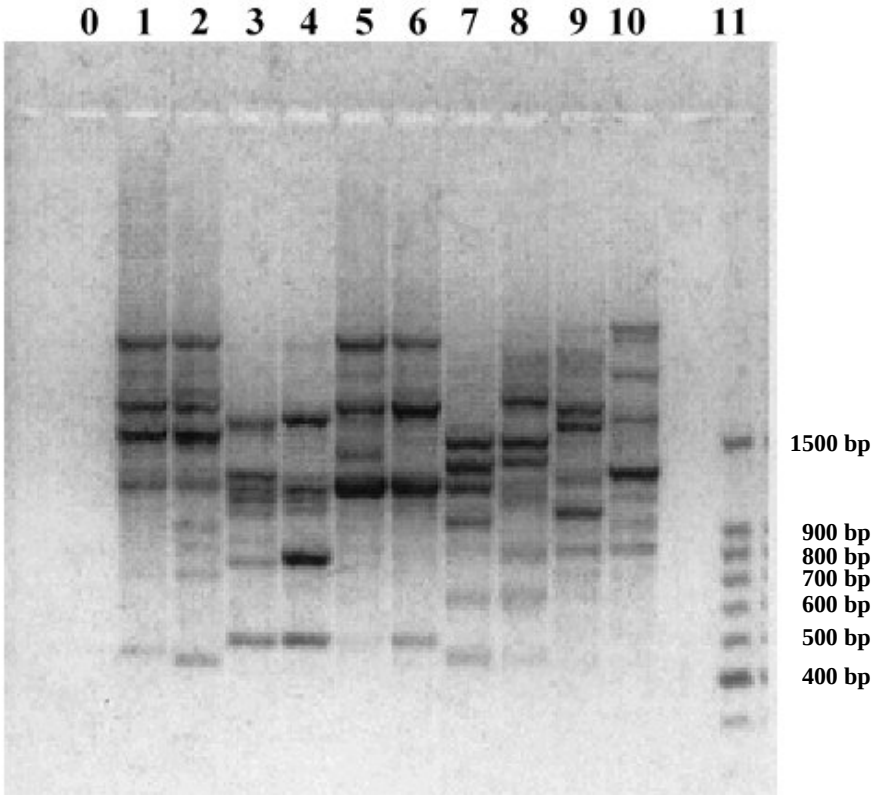

FIGURE 3. A RAPD polymorphism profile generated from primer G-80-23. Lane 0 was negative control, Lanes 1 + 2 *A. bulbosus*; Lanes 3 + 4 *A. aequalis*; Lanes 5 + 6 *A. geniculatus*; Lanes 7 + 8 *A. pratensis*; Lanes 9 + 10 *A. myosuroides*; Lane 11 100 bp (λ) (Promega Ltd) molecular weight marker.

cpDNA parsimony analysis

The single shortest most parsimonious phylogenetic tree of 226 steps is shown in Fig. 5. The observed number of mutations, given as the branch length, was high enough to separate the different taxa analysed, and also to differentiate between species within a genus, e.g. *Spartina*, *Hordeum*, and *Alopecurus*. The chosen cpDNA sequence was thus very informative at all levels. The tree scores were all high; CI (0·858), RI (0·847) and RC (0·727), indicating a low level of homoplasy and therefore high reliability that the data set fit the tree produced. The bootstrap analysis produced values between 70–100% indicating that the tree is robust and the groupings given are statistically reliable. The genus *Alopecurus* form a monophyletic group upheld by a bootstrap value of 99%. Within this clade *A. pratensis* is basal for the genus. There is strong support (92%) for a sub-clade including the three species *A. aequalis*, *A. bulbosus* and *A. geniculatus*. The latter two species had identical $tRNA^{Leu1}$ intron sequences, with the sequence obtained for *A. aequalis* differing from these two by only three mutations.

DISCUSSION

There was a closely correlated relationship between 2C DNA values obtained from the fluorescence intensity of PI stained nuclei and previously published 2C DNA values obtained by Feulgen microdensitometry (R^2 = 0·994, r = (p> 0·05)) (Sieber & Murray *19*79). The 2C DNA values estimated by fluorescence intensity of PI stained nuclei were consistently lower than those

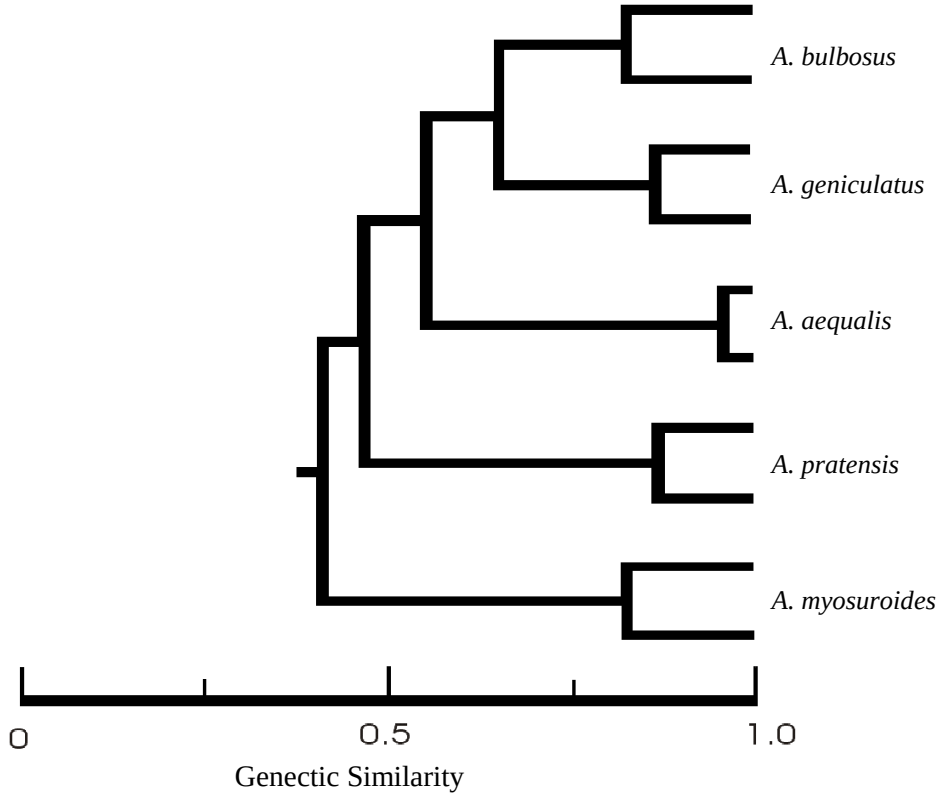

FIGURE 4. Dendrogram of *Alopecurus* spp generated by cluster analysis using genetic similarities generated from 135 RAPD bands produced by 12 primer pairs.

obtained by Feulgen microdensitometry. The discrepancy between estimates of DNA amount of *Alopecurus* by Sieber & Murray (1979) may result from the choice of standards as Sieber & Murray (1979) used *Allium cepa* cv. Suttons A_1 assigning a 2C value of 33·5 pg rather than the recommended standard *A. cepa* cv. Ailsa Craig (Bennett & Smith 1991,1976; Bennett & Leitch 1995; Bennett *et al.*, 2000a). Variation exists between varieties of *A. cepa* (Bennett & Smith 1976) which may account for the differences obtained for 2C values obtained by Sieber & Murray (1979) and those obtained in this study from the fluorescence intensity of PI stained nuclei. By using *A. cepa* cv. Ailsa Craig, Olszewska & Osiecka (1982) obtained a much lower estimate, close to the one obtained here (Table 1). However, Bennett *et al*. (2000b) showed that there was little variation in measurements of the 2C content, by flow cytometry, in either a number of varieties of *A. cepa* or one variety grown at a range of geographical locations. In contrast, Baranyi & Greilhuber (1999) give 2C values for varieties of *A. cepa* ranging from 33·04–35·14 pg DNA with a mean ± S. E. of 34·00 ± 0·14 but attribute much of this variation to a lack of standardisation in the techniques used between different laboratories.

For flow cytometery the calibration standard should ideally have a similar DNA value to that of the species being estimated to minimise technical errors (Bennett & Smith 1976). The 2C DNA amount of *H. vulgare* L. cv. Sultan 2C = 11·12 pg is closer to that of *Alopecurus* than is *A. cepa* (2C = 33·5pg). Use of an internal standard reduces errors resulting from interaction between chromatin and tannins (Greilhuber 1988; Bennett *et al.* 2000a). A further source of variation between the two studies may have been due to differences in DNA content between individual plants used in each study. Specimens were largely collected from the same locations and populations to reduce this variation. However, the composition of the populations studied may

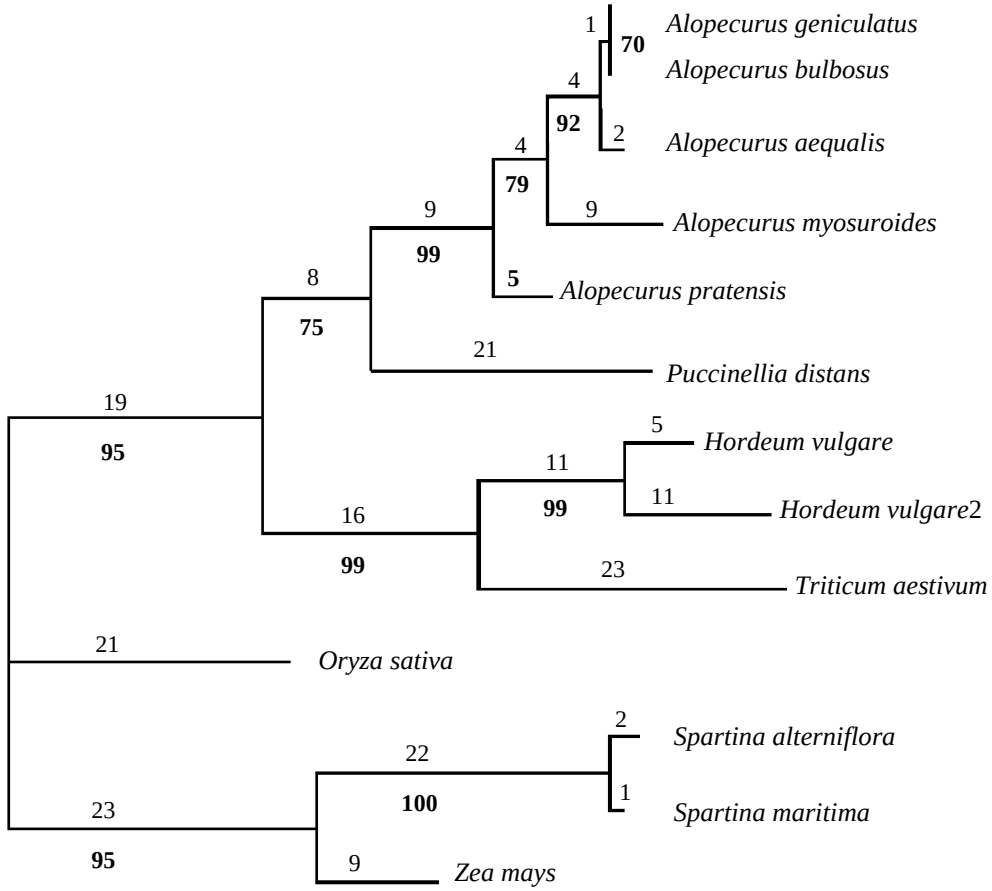

FIGURE 5. The single most parsimonious phylogenetic tree of 224 steps length based on the tRNA^{Leu1} intron sequence data of the five *Alopecurus* species and the tRNA^{Leu1} intron sequences of species from the eight additional Poaceae from the EMBL database. The number of mutations separating each species is given above the branch and bootstrap values are given in bold type below the branch.

have changed during the 20 years separating Sieber & Murray (1979) and the present study. Additional measurements of 2C DNA amount, by Feulgen microdensitometry include: *A. myosuroides* (Bennett & Leitch 1997), *A. pratensis* (Olszewska & Osiecka 1982) and *A. geniculatus* (Bennett & Smith 1991) but are limited as the chromosome number of plants studied was not recorded and the DNA amounts cannot be compared precisely with those of the present investigation.

The DNA amounts in autopolyploids are often a multiplication of the diploid progenitor genome, for example: *Celosia* (Nath *et al.* 1992); *Narcissus*, *Hyacinthus* and *Tulipa* (Brandham & West 1993); *Vaccinium* (Costich *et al.* 1993); *Prunus* (Vance-Baird *et al.* 1994); *Ipomoea* (Ozias-Atkins and Jarret 1994). Allopolyploids may also contain the sum of the 2C contents of their component genomes, for example: *Glycine* (Hammatt *et al.* 1991); *Arachis* (Singh *et al.* 1996); *Allium*, (Ohri *et al.* 1998) and *Salix* (Thibault 1998). Morphologically *A. bulbosus* closely resembles *A. geniculatus* (Hubbard 1984). Sieber & Murray (1979, 1980, 1981) suggested that either *A. bulbosus* or *A. aequalis* might have acted as the diploid progenitor of the *A. geniculatus*. Substantial additional DNA would be required to account for the difference between twice the DNA contents of either *A. aequalis* (2C = 7·20 pg) or *A. myosuroides* (2C = 7·74pg) and the observed 2C DNA content of *A. geniculatus* (16·17 pg). On the basis of 2C DNA values it is possible that *A. geniculatus* originated as a segmental allotetraploid derived from *A. aequalis* and *A. bulbosus*, with loss of a small amount of DNA.

However, meiotic studies of *A. geniculatus* have shown regular multivalent formation, as opposed to the segmental allotetraploid *A. pratensis* that was predominantly bivalent forming, indicating that autotetraploid origins are more likely (Sieber & Murray 1979). Although the frequency of multivalent formation is lower than would be expected from the normal models and formulae for multivalent formation in autopolyploids, it is similar to that found in colchicine induced tetraploids of *A. aequalis* and the low levels multivalent formation in naturally occurring tetraploids of *A. bulbosus* (Murray *et al*.). These studies concluded that the lower levels of multivalent formation seen in *Alopecurus* are the result of genotypic control of preferential bivalent formation (Murray, Sieber & Jackson 1984).

Of the diploid species *A. bulbosus* had the largest 2C DNA content (2C = 9·27 pg) and would appear to be the likely diploid progenitor of *A. geniculatus* if this species is an autotetraploid. However, the nuclear DNA content of *A. geniculatus* is only 87% that of the doubled DNA content of *A. bulbosus*. This suggests that DNA loss may have occurred during the formation of *A. geniculatus*. Evidence for DNA loss during the origin of *A. geniculatus* comes from comparison of the number of NOR sites, the diploid species all have four NOR sites, *A. pratensis* has eight sites whilst *A. geniculatus* has only six sites. This suggests that two NOR sites were lost along with other DNA during the formation of *A. geniculatus*. Loss of NOR sites has been well documented in polyploid species, and physical elimination has been demonstrated by means of *in situ* hybridisation in a number of cases: *Avena sativa* (Jellen *et al.* 1994; Leggett & Markand 1995); *Scilla autumnalis* (Vaughan *et al*. 1993) and *Parnassia palustris* (Wentworth 1995). The loss of rRNA loci might be interpreted as part of the overall diploidisation process that is believed to occur in polyploids (Wendel, 2000; Pikaard, 2001), although the formation of multivalents at a low frequency in *A. geniculatus* (Sieber & Murray 1979) would indicate that this process is incomplete. Loss of DNA during the formation of polyploids has also been described in a range of other genera: *Festuca* (Seal 1983); *Leucanthemum* (Marchi *et al.* 1983); *Bulbine* (Watson 1987); *Pratia* (Murray *et al.* 1992b). Bennett *et al.* (2000a) describe a number of possible mechanisms leading to variation in C-values of polyploid species. Evidence that autopolyploid genomes may experience substantial changes has been reported from artificially produced autotetraploids in *Vicia*, *Tephrosia* and *Phlox*, with the latter eventually losing up to 25% of the expected amount based on its diploid progenitors (Raina *et al*. 1994).

RAPD analysis showed that of these five species the highest degree of genetic similarity occurred between *A. bulbosus* and *A. geniculatus*. However, further studies are required to confirm the genetic similarity between *A. geniculatus* and *A. bulbosus*. Although this study appears to show that there are RAPDs bands that occur only in *A. geniculatus* and *A. bulbosus*, this is also true of *A. geniculatus* and *A. aequalis*. A larger sample size would likely reduce the number of these bands, and the suggested common origin of *A. aequalis* and *A. bulbosus* may mean that number of distinguishing markers would be more limited. The diversity between *A. geniculatus* and *A. bulbosus* also raises some questions over their closeness. The analysis is hampered by the small sample size of two plants, and does not take account of intraspecific variability. In addition, possible complications may have occurred as a result of over assumptions made on band homology. The difficulty of determining the sources of variation between RAPD fragments limits the use of RAPDs in systematics to comparisons between closely related species (Harris 1995; Catalan *et al*. 1995). RAPD fragments separated by electrophoresis are identified by size. However, bands of similar size from different species may not be homologous (Gillies & Abbott 1998). Some authors have shown that in closely related species fragment size is a good predictor of band homology (Reiseberg 1996). Previous studies have suggested that at least four of these five species are closely related (Johnsson 1941; Sieber & Murray 1979, 1980, 1981; Murray, Sieber & Jackson 1984).

Hybridisation between *A. geniculatus* and *A. pratensis* has been found to occur in natural populations with limited fertility amongst the progeny, *A.* × *brachystylus* (Johnsson 1941; Sieber & Murray 1979, 1980). Trivalent formation occurred during meiosis in artificial hybrids between *A. aequalis* and *A. geniculatus* (Johnsson 1941) and *A. aequalis* and *A. pratensis* (Sieber & Murray 1981) suggesting that reproductive isolation between these species results from differences in ploidy rather than lack of genome homology. Evidence from cpDNA, presented in this paper, further confirmed the close relationship between these species, but a systematic survey of RAPD band homology was not undertaken.

Other experimental errors are also likely to have occurred in the RAPDs analysis. Inconsistencies in the data presented here could include scoring errors, competition in the PCR reactions and low reproducibility. RAPD markers are also assumed to be dominant, which limits their usefulness in estimating genetic diversity. As the genetic basis of RAPD markers is poorly understood in most studies based on natural populations, it can be argued that RAPD profiles should be treated as DNA phenotypes rather than genotypes (Harris 1999). Although RAPDs may be suitable for general description purposes, in order to resolve the specific issue of whether *A. bulbosus* is the sole progenitor of *A. geniculatus* it may be necessary to employ other molecular techniques.

The sequencing of the $tRNA^{Leu1}$ intron showed that *A. geniculatus* and *A. bulbosus* had identical $tRNA^{Leu1}$ intron sequences for this region of cpDNA. Although it may be inappropriate to infer the origin of a species from the type of RAPDs data presented here, the combination of RAPDs and chloroplast sequence data indicates that *A. bulbosus* is at least one of the probable diploid progenitors of *A. geniculatus*. It is unlikely that these species would share the same cpDNA as a result of chloroplast capture as the natural hybrid between the two species, *A.* × *plecktii*, is a sterile triploid (Hubbard 1984). Although the differing habitat requirements for *A. aequalis* and *A. bulbosus* would indicate that an allopolyploid origin of *A. geniculatus* as the result of hybridisation between these species is unlikely, confirmation of the autopolyploid origin of *A. geniculatu*s, as indicated by meiotic evidence from previous studies, is still required. This could be achieved by the use of molecular techniques, possibly by a survey of isozymes to establish whether there is a pattern of tetrasomic inheritance in *A. geniculatus*. The construction of a phylogeny using nuclear markers, such as the internal transcribed spacer (ITS) sequences of ribosomal DNA, could also be used to determine whether *A. bulbosus* is the sole progenitor of *A. geniculatus* or if another genome is present.

The RAPD and cpDNA evidence would indicate that *A. aequalis* is closely related to *A. geniculatus* and *A. bulbosus* whilst *A. myosuroides* and *A. pratensis* appear to be more distantly related to them. However, there are differences in the positions of *A. myosuroides* and *A. pratensis* in the RAPDs dendrogram and cpDNA phylogenetic tree presented in Figs 4 & 5. This could be partly a result of the flaws in RAPDs technique outlined above, however, it is most likely the result of data analyses used. The cpDNA analysis parsimony groups according to shared character states, while the UPGMA analysis used similarities. The short branch length between *A. pratensis* and *A. myosuroides* on the UPGMA tree is weakly upheld whereas the branching pattern on the cpDNA tree is upheld by high bootstrap values. This takes into account other members of the Poaceae suggesting the cpDNA phylogenetic tree is likely to be the most accurate.

Spontaneous formation of triploid and tetraploid individuals in natural populations of *A. bulbosus* (Sieber & Murray 1980) supports the hypothesis that this species could be the sole progenitor of *A. geniculatus*. The formation of autopolyploids from unreduced gametes has been shown to occur in a wide range of plant species (De Wet 1980; Bretagnolle & Thompson 1995; Ramsay & Schemske 1998). Establishment and survival of newly formed polyploids is increased in small populations, where genetic inbreeding and environmental factors cause an increase in diploid gamete formation (Fowler & Levin 1984; Thompson & Lumaret 1992). Ecological preferences may separate newly formed polyploid individuals from their diploid progenitor, reducing competition and hybridisation (Lumaret *et al*. 1987; Lumaret 1988). *A. bulbosus* is a rare perennial, which occurs in small populations on the margins of salt-marshes in southern England. *A. geniculatus* is reproductively isolated from *A. bulbosus* as the naturally occurring hybrid *A.* × *plecktii* is sterile (Hubbard 1984). There appears to be strong niche differentiation between *A. bulbosus* and *A. geniculatus* in that the species maintain strict zonation, as *A. bulbosus* tolerates salinity and does not grow in the wet areas usually occupied by *A. geniculatus* (Fitzgerald 1989). The triploid hybrid, *A.* × *plecktii*, may under some conditions be at a competitive advantage to *A. bulbosus* but appears to be unable to compete under conditions of increasing salinity and is sterile, promoting niche differentiation between *A. bulbosus* and *A. geniculatus*.

This study presents both indirect and direct evidence that a diploid species of limited distribution, *A. bulbosus*, is one, or the sole progenitor, of the widely distributed tetraploid species *A. geniculatus*. The indirect evidence for this hypothesis is provided by the DNA content of *A. geniculatus*, which is 87% of that of the doubled genome of *A. bulbosus*, implying that DNA loss has occurred following the duplication of the genome. Evidence of DNA loss is further indicated

by the occurrence of only six NOR sites instead of the eight NOR sites that would be expected. RAPD analysis indicates that of the five species in the study, *A. bulbosus* and *A. geniculatus* have the highest similarity, although as outlined above this data is not conclusive. CpDNA analysis does, however, show that *A. bulbosus* and *A. geniculatus* have identical haplotypes, suggesting that *A. bulbosus* was a diploid progenitor of *A. geniculatus* via auto- or allopolyploidy. Previous meiotic studies have indicated an autopolyploid origin for *A. geniculatus*, but further research is required to provide a definitive answer, possibly by the sequencing of the rDNA ITS regions. It would also be constructive to extend the cpDNA phylogeny and any proposed ITS based phylogeny beyond these five species to include all the diploid species in the genus. This could identify or exclude any species that are implicated in an allopolyploid origin event for *A. geniculatus*.

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