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Phylogenetic analysis in the *Festuca-Lolium* complex using molecular markers and ITS rDNA

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Abstract Molecular markers were used to investigate phylogenetic relationships among the eight species of ryegrass (*Lolium*) and 11 species of fescue (*Festuca*). RAPD and RFLP analyses were carried out on total bulked DNA from each population. Factorial analysis of a phenetic distance matrix yielded three major groups: (1) fine-leaved fescues, (2) broad-leaved fescues and (3) ryegrasses. Six non-coding regions of chloroplastic DNA were PCR-amplified, then digested by 20 restriction enzymes. Nuclear rDNA sequences, including internal transcribed spacers (ITSs) were used to estimate the average proportion of nucleotide substitutions. The correlation between substitution rate estimated from ITS sequences and that estimated from organelle DNA restriction sites was very high (0.94), and the corresponding UPGMA trees were very similar, with a slightly better resolution of the ITS tree in the *Lolium* genus. The time-scale inferred from substitution rates indicated that the period since divergence of the broad-leaved fescues from the fine-leaved fescues was four times as long as that since divergence of the genus *Lolium* from the former. Among the broad-leaved fescues, meadow fescue was closer to the *Lolium* group, while *F. glaucescens* and tall fescue were very closely related. North-African fescues were clustered together and giant fescue was the most differentiated species in this group. Our dataset was merged with ITS sequences recovered from the EMBL database, and the neighbor-joining method was used to draw a phylogenetic tree. In this tree, the tribe Poeae was clearly monophyletic, and more closely related to the Aveneae than to the Triticeae or Bromoideae. The genus *Festuca* appeared somewhat artificial, since *Vulpia myuros* and *Dactylis glomerata* were placed between fine-leaved and broad-leaved fescues.

Key words *Lolium* · *Festuca* · Phylogeny · Chloroplast DNA · ITS sequence · Genetic distance · Evolution

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Introduction

The genus *Festuca* L. is one of the largest in the Poaceae (grass) family with more than 400 species (Clayton and Renvoize 1986). Several species of important agronomic use in temperate countries are found in two main subgenera: (1) broad-leaved fescues (subg. *Schedonorus*) including meadow fescue (*F. pratensis*) and tall fescue (*F. arundinacea*) which are found in natural and sown grassland, (2) fine-leaved fescues (subg. *Festuca*) comprise red fescue (*F. rubra*) and the *F. ovina* group which occur in natural meadows and turf lawns. The related genus *Lolium* includes two species used worldwide, namely perennial ryegrass (*L. perenne*), for grazing and turf, and Italian ryegrass (*L. multiflorum*), for grazing or hay and silage making. Other uses such as soil preservation or nitrate capture are also under development. All the *Lolium* species are diploid with $2n = 14$ chromosomes (Terrell 1968), whereas the genus *Festuca* is a polyploid complex with 2n ranging from 14 to 70 (Borril et al. 1971).

A better knowledge of phylogenetic relationships among the *Festuca*/*Lolium* species would be very useful for the collection, conservation, and use of wild species related to cultivated types as genetic resources. Until the 1990s, most knowledge on the taxonomy and phylogenetic relationships of the *Festuca*/*Lolium* complex came from cytological studies and interspecific crosses (e.g. Terell 1966; Malik and Thomas 1967; Chandrashekharan and Thomas 1971). More recent work has used morphological and seed-protein analyses (Bulinska-Radomska and Lester 1988), cpDNA

restriction-site analysis (Lehvaslaiho et al. 1987; Darbyshire and Warwick 1992; Yaneshita et al. 1993), RFLP (Xu and Sleper 1994) or RAPD (Stammers et al. 1995) markers. However, with the exception of the RAPD study of Stammers et al. (1995), most of these studies included a large sample of *Festuca* but only a few *Lolium* species. In previous papers (Charmet and Balfourier 1994; Charmet et al. 1996), we described the phylogenetic relationships between all eight species of *Lolium* using isozyme frequency. The aim of the present paper was to extend this study to some representative species of the genus *Festuca*, and to use more powerful tools, namely RFLP and RAPD markers, restriction-site analysis of non-coding cpDNA, and ITS sequences of nuclear rDNA, for inferring phylogenetic trees.

Material and methods

Twenty nine natural populations belonging to the eight species of *Lolium* and to 11 species of *Festuca*, including those of importance in agriculture, as well as one species of *Vulpia* and one of *Poa*, were used for DNA marker analyses (Table 1). In addition, ITS DNA sequences of some other species of temperate grasses of European origin were recovered from the "EMBL" database via internet. Although the origin of these data is anonymous, they are likely to be those published by Hsiao et al. (1995).

A bulk of at least 50 seedlings was used to extract total DNA by the CTAB method. Four types of molecular analyses were carried out on these DNA samples: RAPD and RFLP of total DNA, RFLP of specifically amplified regions of cpDNA, and sequencing of internal transcribed spacers (ITSs) of nuclear ribosomal DNA.

RFLP of total DNA

The procedures for RFLP analysis were performed with digoxigenine as described in Lu et al. (1994). Fourteen wheat gDNA probes (kindly provided by Dr. Nelson, Cornell University, USA, and by the Génoblé club of French wheat breeders) and 13 perennial ryegrass cDNA probes (kindly provided by Dr. M. D. Hayward, AFRC Aberystwyth, UK)) were hybridized with DNA digested with four 6-base restriction enzymes: *Eco*RI, *Eco*RV, *Hin*dIII and *Dra*I. Owing to the genetic heterogeneity of bulked DNA from cross-fertilized populations, multiple banding patterns were expected, even for single-copy probes, with the intensity of the band being proportional to the allele frequency. However, as differences in intensity were difficult to quantify, we simply recorded the presence/absence of bands.

RAPD analysis

The same DNA samples were used for random amplification according to Stammers et al. (1995). Twenty two 10-mer primers (Operon technology, most in kit C) were used and yielded 422 bands. As described for RFLP, the presence/absence of bands showing "good" intensity was recorded.

Table 1 Description of the genetic material used in this study

Accession	Entry code	Genus	Subgenus	Species name	Ploidy	Genomes	Origin
1	11 118	*Lolium*		*perenne*	2x	L	France
2	210 007	*Lolium*		*perenne*	2x	L	Bulgaria
3	30 018	*Lolium*		*multiflorum*	2x	L	Portugal
4	40 032	*Lolium*		*multiflorum*	2	L	Italy
5	120 028	*Lolium*		*multiflorum*	2x	L	Germany
6	11 460	*Lolium*		*rigidum*	2x	L	Corsica
7	40 126	*Lolium*		*rigidum*	2x	L	Italy
8	330 009	*Lolium*		*rigidum*	2x	L	Tunisia
9	610 008	*Lolium*		*temulentum*	2x	L	Tunisia
10	620 001	*Lolium*		*remotum*	2x	L	France
11	630 001	*Lolium*		*persicum*	2x	L	Iran
12	640 001	*Lolium*		*subulatum*	2x	L	Greece
13	20 501	*Lolium*		*canariense*	2x	L	Tenerife
14	20 516	*Lolium*		*canariense*	2x	L	Gomera
15	650 005	*Festuca*	*Schedonorus*	*pratensis*	2x	P	France
16	650 008	*Festuca*	*Schedonorus*	*pratensis*	2x	P	G. Britain
17	680 001	*Festuca*	*Schedonorus*	*glaucescens*	4x	G1 G2	France
18	660 001	*Festuca*	*Schedonorus*	*arundinacea*	6x	P G1 G2	France
19	660 008	*Festuca*	*Schedonorus*	*arundinacea*	6x	P G1 G2	France
20	660 010	*Festuca*	*Schedonorus*	*mairei*	4x	M1 M2	Morocco
21	660 011	*Festuca*	*Schedonorus*	*atlantigena*	8x	G1 G2 M1 M2	Morocco
22	660 012	*Festuca*	*Schedonorus*	*letourneuxiana*	10x	Q G1 G2 M1 M2	Morocco
23	690 006	*Festuca*	*Schedonorus*	*gigantea*	6x		France
24	670 002	*Festuca*	*Festuca*	*rubra*	6x		France
25	670 010	*Festuca*	*Festuca*	*heterophilla*	4x		France
26	670 011	*Festuca*	*Festuca*	*ovina*	6x		France
27	670 012	*Festuca*	*Festuca*	*filiformis*	2x		France
28	700 002	*Poa*		*trivialis*	2x		France
29	720 001	*Vulpia*		*myuros*	6x		France

RFLP of cpDNA

Six pairs of 20-mer primers were used to amplify specifically non-coding regions of chloroplastic DNA (Taberlet et al. 1991; Demesure et al. 1995). PCR was performed using a Perkin-Elmer thermocycler, according to the protocol described by Demesure et al. (1995). Amplified fragments were then digested overnight using 20 restriction enzymes (10 units/10 µl of amplification product): 12 with 6-base recognition sites: *Bam*HI, *Bgl*II, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I, *Sty*I, *Xba*I, *Xho*I; and eight with four-base recognition sites: *Alu*I, *Cfo*I, *Dde*I, *Hae*III, *Hin*fI, *Msp*I, *Rsa*I, *Taq*I. Digestion products were analysed by electrophoresis in 2% agarose gels run for 4–5 h at 2.5 $V\,cm^{-1}$ and stained with ethidium bromide. The presence/absence of restriction sites was coded for each population.

ITS region sequencing

The entire region of nuclear ribosomic DNA which comprises both internal transcribed spacers ITS1 and ITS2 and the 5.8*s* subunit was PCR-amplified using primers ITSL and ITS4, which are complementary to 18*s* and 26*s* rDNA near the ITS1 and ITS2 borders, respectively (Hsiao et al. 1995). Direct double-stranded DNA sequencing was carried out by GENOME EXPRESS (Grenoble, France). The sequencing reaction was performed by PCR amplification in a final volume of 20 µl using 100 ng of PCR products, 5 pmoles of primer, and 9.5 µl of DyeTerminators premix according to the Applied Biosystems protocol. After heating to 94°C for 2 min, the reaction was cycled as follows: 25 cycles of 30 s at 55°C and 4 min at 60°C (9600 thermal cycler Perkin Elmer). Removal of excess of DyeTerminators was performed using Quick Spin columns (Boehringer Mannheim). The samples were dried in a vacuum centrifuge and dissolved with 4 µl of de-ionized formamide EDTA pH 8.0 (5/1). Samples were loaded onto an Applied Biosystems 373A sequencer and run for 12 h on a 4.5% denaturing acrylamide gel.

Statistical analyses

All data were first transformed into estimates of genetic distance. Among the range of distance formulae available, we used the simplest and the most widely used, that of Nei and Li (1979), which is 1 minus the proportion of shared bands between each pair of populations. We checked that the distance estimator has very little influence on the phylogenetic reconstruction. Pearson correlation coefficients were computed among various distance matrices. This statistic is equivalent to the normalized Mantel test, and must obviously be tested by the same procedure of random permutations (Mantel 1967), because distances in a matrix are not independent of each other. Pairwise distance matrices were summarized using multi-dimensionnal scaling plots.

As chloroplast DNA (cpDNA) restriction data showed no variation within populations (checked on ten plants from three populations), the banding patterns were interpreted in terms of presence/absence of restriction sites. Given the length of restriction sites, it was then possible to infer the average proportion of nucleotide substitutions, using the maximum likelihood formula of Nei and Tajima (1983), which takes into account the length of recognition sites for each class of enzyme. As the assumption of a constant rate of substitution among all species seemed to be reasonable for non-coding sequences of cpDNA, the classical UPGMA method, which implicitly makes this assumption, was used to obtain a phylogenetic tree from the DNA restriction data.

For ITS sequence analysis of our own dataset, the Kimura (1981) two parameter estimate of average nucleotide substitution was computed using PHYLIP software (Felsenstein 1993) and an UPGMA tree was drawn from the ITS-derived distance matrix of the 27 *Lolium*/*Festuca* populations. In order to avoid the assumption of a constant rate of nucleotide substitution, which was less obvious for a more diversified group than our own subset, the neighbor-joining method (Saitou and Nei 1987) was used on the enlarged ITS dataset which included sequences recovered from the EMBL database, using *Brachypodium pinnatum* as an outgroup, according to Hsiao et al. (1995). The robustness of nodes in the tree was tested by bootstrap re-sampling as proposed by Felsenstein (1985).

Results

RFLP and RAPD

Five hundred and sixty four RFLP bands were obtained with 27 probes, i.e. an average of 20.9 bands per probe, with no difference between wheat gDNA and ryegrass cDNA probes. It should be noted that this large number of bands is due to the wide range of variability among the set of species studied, and to the genetic heterogeneity of the grass population. The average number of bands per population is around eight and, when hybridized to single-plant DNA, most probes gave clear patterns with 2–3 bands (data not shown). Quite similar figures were obtained for RAPDs with a mean number of bands per primer of 19.2.

The pairwise Nei and Li similarity matrices from RFLP or from RAPD data were highly correlated (0.91). Therefore data from RFLP and RAPD were merged together to yield a unique dataset of 986 bands. Bootstrap re-sampling procedures as described by Tivang et al. (1994) were used to estimate the coefficient of variation of genetic similarity as a function of band sample size. With the full set of data, genetic similarity is estimated with a coefficient of variation of about 8%, the 10% threshold being reached with around 600 bands in the subsample. Very similar patterns were obtained for RAPD or RFLP bands used separately. When a species is represented by several populations, the within-species distance among populations is always smaller than the distance from the closest species, with at least 80% of shared bands. Figure 1 shows the first plan of the multi-dimensional scaling of the distance matrix, which accounts for 73% of the inertia. Three major groups clearly appear on the dendrogram: (1) fine-leaved fescues and outgroup species, (2) broad-leaved fescues, and (3) ryegrasses. In more detail, it should be noted that the *Lolium* group is split into a group of self-fertilized and a group of open-pollinated species, and that *F. pratensis* seems to be the *Festuca* species most closely related to the genus *Lolium*.

Restriction analysis of cpDNA

Overall, 213 restriction sites were obtained, among which 67 were common to all species and 146 were informative (Table 2). If sites do not overlap, this represents a surveyed sequence of 992 nucleotides. The

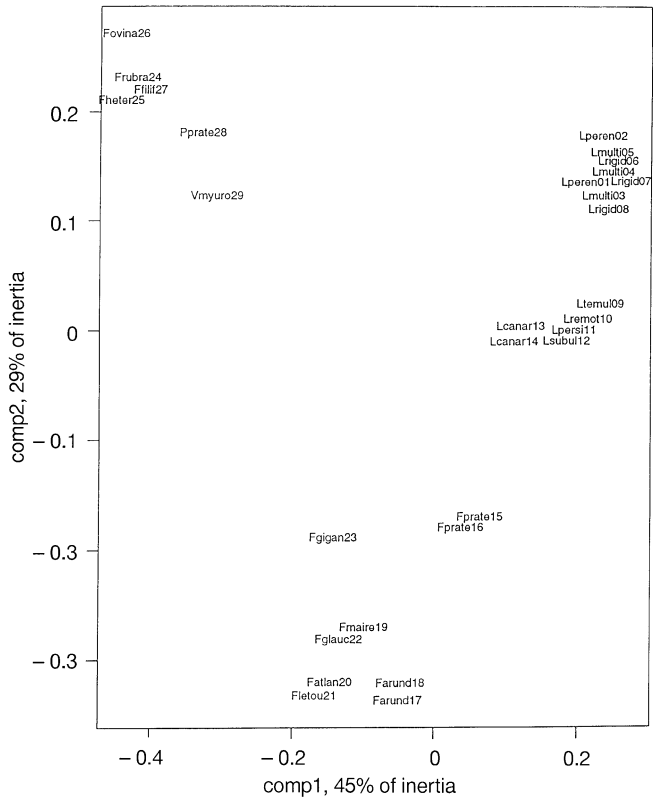

Fig. 1 Multi-dimensional scaling plot of the Nei and Li pairwise distance matrix among the 29 populations

average proportion of nucleotide substitutions ranges from 0 within some species, or even between species in the self-fertilized *Lolium* group, to 0.145 between *Lolium perenne* and *Poa trivialis*, which are the most distantly related species. The UPGMA dendrogram is presented in Fig. 2 a. Assuming a molecular-clock hypothesis, which seems reasonable for non-coding cpDNA, it can be regarded as a phylogenetic tree. While *P. trivialis* L. clearly lies outside the *Festuca-Lolium* complex, which could thus be considered as a monophyletic group, *Vulpia myuros* shows some affinities with the fine fescues of the subgenus *Festuca*. The three groups of the phenetic analysis appear again, with the exception of *F. pratensis* which is closely related to the genus *Lolium*.

Sequence analysis of ITS DNA

The complete sequences of a 647-nucleotide region, including ITS1 and ITS2 spacers and the 5.8*s* coding region, were obtained from the amplification products of 27 out of 29 populations, but failed for *F. gigantea* and *F. ovina*. The average nucleotide substitution rate estimated with Kimura's two-parameter model correlates very closely ($r = 0.94$) with that estimated from cpDNA restriction sites, and the corresponding UPGMA tree is therefore very similar to the former (Fig. 2b). Our own ITS data were merged and aligned with ITS sequences recovered from the EMBL database. The comparison of a 630-nucleotide common fragment allowed us to estimate the average susbtitution rate, which reached 0.28 for the most distantly related species. The phylogenetic tree obtained by the neighbor-joining method using *B. sylvaticum* as an outgroup is shown in Fig. 3. In this tree, the tribe Poeae is clearly a monophyletic group, which is more closely related to the tribe Avenae than to the Bromeae or the Triticeae. Most nodes appear to be robust through bootstrap re-sampling. The main uncertainties lie in the genus *Lolium* for *L. cararíense* and *L. temulentum*, which would better be expected in the self-fertilizing group, and for *Dechampsia cespitosa*. If the position of *Dactylis glomerata* is not a sampling artifact, it would point to the artificial origin of the "genus" Festuca, as it lies between the fine-leaved "*Festuca*" subgenus and the broad-leaved "*Schedonorus*".

Discussion and conclusion

Molecular markers such as RFLP of total DNA have been widely used for reconstructing phylogenetic relationships from the overall genome similarity (e.g. Song et al. 1988; Debener et al. 1990; Miller and Tanksley 1990). A similar study was reported for tall fescue and related species by Xu and Sleper (1994), who found that *F. pratensis* clustered with Lolium, *F. glaucescens* with *F. arundinacea*, and the polyploid series of North African fescues with *F. mairei*. It should be noted that some *Lolium* probes did not hybridize to the most distantly related species, such as *P. trivialis* or the fine-leaved

Table 2 Summary of results on cpDNA amplification and restriction

DNA region	Fragment length (bp)	No. restriction sites for 6-base enzymes	No. restriction sites for 4-base enzymes	Sequence length surveyed
trnH-trnK	1690	9	19	130
trnS-trnT	1580	7	20	122
trnT-trnF	1700	12	17	140
trnM-rbcL	2900	15	33	222
rbcL-Orf106	2100	16	25	196
psbC-trnS	1680	11	29	182
Total		70	143	992

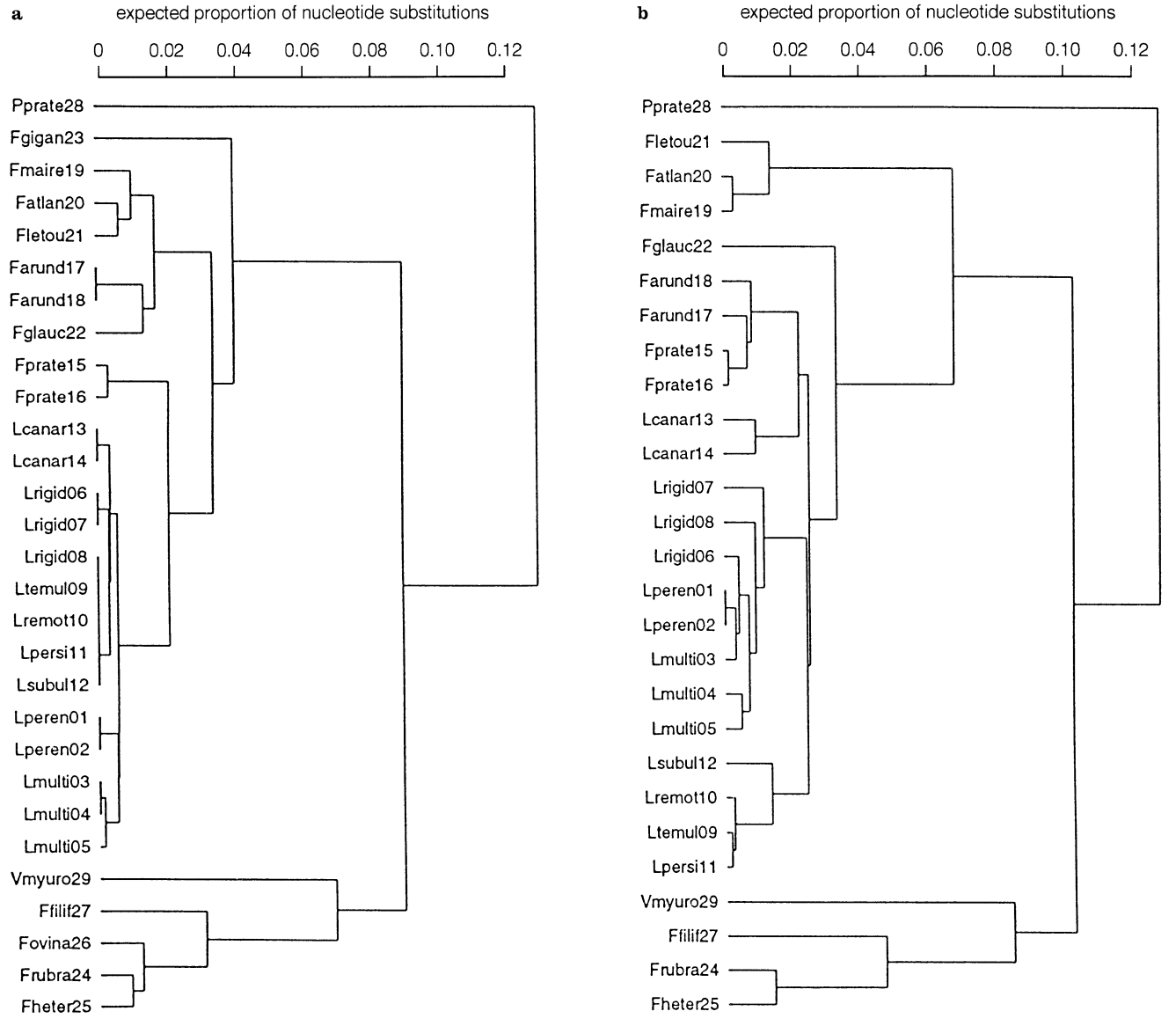

Fig. 2 UPGMA dendrograms from the average nucleotide substitution estimated from **a** cpDNA restriction-site data; **b** ITS sequences

fescues. This resulted in an artifactual lack of shared bands, which led us to underestimate the genetic similarity among the most distant species. However, it can be considered that, although these missing data affect the absolute value of genetic similarity, the clustering of species is not much changed.

The use of RAPD markers in phylogenetic studies has also been reported for a range of species (e.g. Demeke et al. 1992) and for the *Lolium/Festuca* complex by Stammers et al. (1995). The genetic validity of RAPD markers has been questioned, because the homology of RAPD bands of the same molecular weight is uncertain. However, several authors (Thormann et al. 1994; Lanner et al. 1996) have checked the homology of RAPD bands by hybridization with RAPD fragments used as probes and found low error rates, which are not likely to significantly affect estimates of genetic relatedness. RFLP and RAPD markers can therefore be considered as providing similar information about genomic relations between species. In the present study, both RFLP and RAPD on bulked total DNA, as proposed by Yu and Pauls (1993), proved an efficient tool in estimating overall genetic similarities among grass populations, and gave highly correlated estimates of genetic distances. Moreover, bootstrap re-sampling from the combination of RAPD and RFLP bands provided fairly good coefficients of variation.

In the present study, phenetic analysis of the similarity matrix based on both RFLP and RAPD markers succeeded in identifying the three major taxonomic groups: (1) fine leaved fescues, (2) broad-leaved

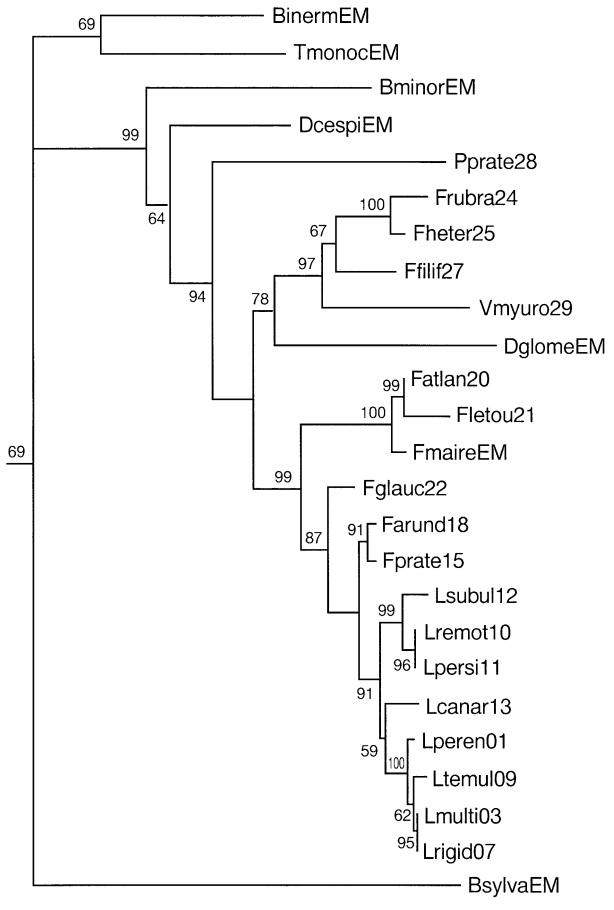

Fig. 3 Neighbor-joining phylogenetic tree of *Festuca/Lolium* and other Poaceae species from ITS sequences of the present experiment merged with those of the EMBL database. Data from EMBL are: *BinermEM*: *Bromus inermis*; *TmonocEM*: *Triticum monococcum*; *BminorEM*: *Briza minor*; *DcespiEM*: *Deschampsia cespitosa*; *DglomeEM*: *Dactylis glomerata*. *Bold figures* are the percent of occurrence of nodes in 200 bootstrap re-samplings

fescues and (3) ryegrasses. In the broad-leaved fescues, all "tall fescues", both European and North African, are very closely related, while *F. gigantea* is slightly differentiated from tall fescue, and meadow fescue tends to be intermediate with ryegrasses. In *Lolium*, there is a clear differentiation between self-and cross-pollinated species. These results are similar to those of previous studies on the *Lolium/Festuca* complex using RFLP (Xu and Sleper 1994) or RAPD (Stammers et al. 1995). However, we did not use UPGMA clustering on these data, because the assumption of a "molecular clock" can hardly be made for RFLP and RAPD.

Organelle DNA has also been extensively used in the past decade for phylogenetic studies (see Olmstead and Palmer 1994 for a review). A first approach for organelle DNA analysis is restriction-site mapping of the whole DNA (e.g. Palmer and Zamir 1982). This was mostly applied to cpDNA, because its size and structure is highly conserved, whereas mitochondrial DNA is known to evolve rapidly in size and structure, but slowly in sequence (Palmer and Herbon 1988), which makes its use in phylogenetic studies more difficult than that of cpDNA. Applications of cpDNA restriction mapping to forage grasses have been reported by Lehväslaiho et al. (1987), Soreng et al. (1990), Darbyshire and Warvick (1992), and Yaneshita et al. (1993).

Another method of analysing cpDNA is the study of homologous fragments amplified by PCR using specific primers. The primer sequences, taken from highly conserved zones, may amplify either coding regions (genes) or non-coding regions (introns or spacers). This method avoids the specific extraction of cpDNA and, as it compares homologous regions of nearly constant size, it makes it possible to obtain more reliable estimates of average nucleotide substitution, which are very valuable data for phylogenetic reconstruction. These substitutions can be estimated either by direct sequencing of the amplified fragment, or can be inferred from restriction-site data. As the rate of nucleotide substitution is 3–10-times higher for non-coding versus coding regions (Wolfe et al. 1987; Giely and Taberlet 1994), these latter will be preferred for the study of distantly related species (e.g. Doebley et al. 1990), while the former are mostly useful for closely related species (e.g. Ogihara et al. 1991).

Our estimates of substitution frequency among species are of the same magnitude as those reported by Lehvaslaiho et al. (1987). Assuming the constancy of nucleotide substitution rate over all cpDNA spacers and over all lineages (molecular-clock hypothesis), the phenetic UPGMA dendrogram can be regarded as a phylogenetic tree. Under this assumption, this method has been demontrated to be efficient for recovering the "true" phylogenetic tree in simulation studies (Sourdis and Krimbas 1987) Moreover, the nucleotide substitution frequency can be converted into a time-scale by $t = d/2\alpha$. Estimates of α, the constant rate of nucleotide substitution in cpDNA spacers, are not easy to find in the literature. Values of $3–5 \times 10^{-9}$ are reported by Wolfe et al. (1987) for distantly related species such as spinach/tobacco, while data from Ogihara et al. (1991) in Triticeae grasses allowed a calculation of the α value of 3.75×10^{-9} from wheat/rice divergence and of 1.33×10^{-8} for *Triticum aestivum/Aegilops crassa*. Taking 5×10^{-9} as a rough and easily tractable estimate of α, the sequence of evolutionary events shown in this tree are: (1) the sub-family *Festuceae* diverged from *P. trivialis* some 13 million years (My) ago, then (2) spilt into broad-leaved and fine-leaved fescues (this last group including *Vulpia*) by about 9 My, finally (3) in the broad-leaved group, the genus *Lolium* diverged around 2 My ago, and its differentiation into species is very recent, about 1 My ago, a value which is similar to that reported for differentiation in the genus *Triticum* (Stebbins 1981). It should be kept in mind that this evolutionary history is that of

cytoplasm, i.e. the maternal lineage of a species. In some instances, this may allow inferences on the origin of the cytoplasm in the interspecific crosses which gave rise to the polyploid series of *Festuca*. As an example, *F. arundinacea* and *F. glaucescens* seem to share a more similar cytoplasm than that of *F. pratensis*, indicating that *F. glaucescens* (or an ancestor close to it) was the female parent in a cross with *F. pratensis* (Humphreys et al. 1995).

The most reliable tool for phylogenetic analysis is direct sequencing of DNA. Ribosomal DNA (rDNA) has been used for a broad range of phylogenetic studies (Hamby and Zimmer 1992). In particular, the internal transcribed spacer regions (ITSs) are more divergent than coding rDNA in their nucleotide sequences, although relatively conserved in length, and are well suited for intra-generic studies (e.g. Baldwin 1992). The application of ITS sequence analysis in grasses has been reported by Hsiao et al. (1994, 1995) and Sun et al. (1994).

The UPGMA tree derived from ITS nucleotide-substitution analysis of our own set of data is very similar to that obtained from organelle DNA. The average nucleotide substitution between fine-leaved and broad-leaved fescue groups is around 0.10, which indicates that both nuclear or chloroplastic non-coding DNAs evolve at a similar rate, perhaps slightly faster for ITS sequences. The main difference compared to the cpDNA tree, is that North-African tall fescues, derived from *F. mairei*, have diverged from the rest of the group long before the differentiation of the genus *Lolium*. This discrepancy may be partially explained by considering *F. glaucescens* as the maternal parent of North African *F. atlantigena* and *F. letourneuxiana*, thus leading to a close grouping with other broad-leaved fescues on the cpDNA tree, while *F. mairei*, one of the genomic components of North African fescues, diverged earlier.

The phylogenetic trees of the *Festuca*/*Lolium* complex obtained from estimates of nucleotide substitutions of either cpDNA or rDNA spacers are highly congruent. They both show the same figure for the differentiation of the three major groups mentioned above. The relative position of species among these major groups is consistent with previous studies including the same species, most of them based on cpDNA (Lehvaslaiho et al. 1987, Darbyshire and Warvick 1992; Yaneshita et al. 1993). The ITS-derived tree displays a better resolution, particularly for the divergence of *Lolium* species, while several of them have identical cpDNAs. In the ITS tree, the first differentiation in the genus *Lolium* was between the outbred and the self-fertilized group, which confirms data from isozymes (Charmet and Balfourier 1994). Among the outbred species, perennial ryegrass seems to have differentiated last, although the resolution of the branching events is probably not sufficient for an average substitution rate of about 1%.

When including ITS sequence data from other sources, we use the neighbor-joining (NJ) method (Saitou and Nei 1987) instead of the classical UPGMA, in order to avoid the assumption of the constancy of substitution rate. Under this condition, the NJ method has been shown to be more efficient than UPGMA for recovering the "true" phylogenetic tree from simulated datasets (Saitou and Nei 1987; Sourdis and Krimbas 1987), and among the most efficient when compared to other distance-based methods or to parsimony methods (Sourdis and Nei 1988; Saitou and Imanashi 1989; Jin and Nei 1991). Even with a relatively small sequence, most nodes appear to be reasonably robust through bootstrap re-sampling. The general classification of this set of grasses is very consistent with that of Hsiao et al. (1995). The genus *Festuca* appears to be a polyphyletic clade, considering the high level of divergence between fine-leaved and broad-leaved groups. The genera *Vulpia* and *Dactylis* seem to be included in the genus *Festuca*, although the node is less certain for *Dactylis*. This agrees with an old classification of Hackel (cited in Darbyshire and Warwick 1992), who proposed *Vulpia* as a subgenus of *Festuca*. Similarly, the intermediate position of *Dactylis* between fine-leaved fescues (subgenus *Festuca*) and broad-leaved fescues (subgenus *Schedonorus*) was reported by Leväslaiho et al. (1987), and Darbyshire and Warwick (1992) even reported *Poa pratensis* and *Puccinelia distans* to be in a similar position in their UPGMA tree from cpDNA restriction sites. These results are also consistent with data from cytological (Borril et al. 1977) and biochemical studies (Butkute and Konarev 1982; Bulinska-Radomska and Lester 1988). Within the subgenus *Schedonorus*, tall fescue and meadow fescue are closely related to each other and to the *Lolium* species, which is again consistent with previous studies, while all the North African polyploid species derived from *F. mairei* form a distinct group which diverged earlier from *F. arundinacea* and *F. pratensis*. The eight *Lolium* species appear to be of recent origin and, according to the results of Stammers et al. (1995), we found that the self-fertilizing species diverged first from the common ancestor and the outbred species last, contrary to the hypothesis of Thomas (1981) from C-banding karyotypes.

In conclusion, the phylogenetic interpretation of different categories of molecular markers gave very convergent results, as reported by Spooner et al. (1996) for *Solanum*. Our results are consistent with most of the previous reports in the literature. The molecular-clock hypothesis allowed us to roughly estimate the time of divergence among the main taxonomic groups. It should be particularly stressed that the genus *Lolium* is of very recent origin, and its interior nodes are consequently not resolved with certainty. A practical consequence of this is that the genus *Lolium* should be regarded as a single entity as far as its use as a genetic resource for the breeding programmes of cultivated

species is concerned. This classification of the subtribe Festuceae will also be used in further studies for a comparison with the classification of *Neotyphodium* (formerly *Acremonium*), their endophytic fungi.

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