Theor Appl Genet (1994) 88: 685–690

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Phylogeny of tall fescue and related species using RFLPs

Received: 18 October 1993 / Accepted: 26 October 1993

Abstract The wild species of tall fescue (*Festuca arundinacea* var. *genuina* Schreb.) represent a wide range of genetic variation and constitute potential germplasm for tall fescue improvement. Our objective was to evaluate genome specificity of the previously-identified DNA probes and to examine the phylogenetic relationship of tall fescue with six related species by using RFLP data. A total of 29 DNA probes from a *Pst*I-genomic library of tall fescue were hybridized to *Eco*RI- or *Hin*dIII-digested DNA of 32 plants from six *Festuca* species and from *Lolium perenne* L. Fifteen probes hybridized to all seven species. The remaining 14 probes showed differential hybridization patterns (i.e., ±), especially at the diploid and tetraploid levels. This hybridization pattern reflected genome divergence in these species. The DNA probes will be useful markers in breeding programs involving interspecific and intergeneric hybridization. Cluster analyses were performed using the average genetic distances calculated with the RFLP data from 53 probe-enzyme combinations. Generally, genotypes from the same species were grouped in the same cluster. These data indicated that tall fescue has a close relationship with *F. pratensis* Huds. (diploid), *F. arundinacea* var. *glaucescens* Boiss. (tetraploid), and *L. perenne* L. (diploid) and that *Festuca pratensis* and *L. perenne* had the closest degree of relationship.

Key words *Festuca arundinacea* · *Lolium perenne* Genome-specific probes · Grass breeding

Introduction

A few of the approximately 80 *Festuca* species are diploids; the majority are highly polyploid. The most widely-cultivated species is the perennial and cross-pollinating tall fescue. Cytogenetic studies showed that tall fescue is an allopolyploid (2n=6x=42) and originated from *F. pratensis* (2n=2x=14) and *F. arundinacea* var. *glaucescens* (2n=4x=28) (Sleper 1985). The *F. pratensis* genome is designated as P and the two *F. arundinacea* var. *glaucescens* genomes as G_1 and G_2. Lewis et al. (1980) found that the inheritance of the isozyme phospho-glucoisomerase locus 2 was disomic and chromosome pairing was restricted to true homologues in tall fescue.

Tall fescue and its two putative progenitor species are morphologically similar, as with the octoploid *F. arundinacea* var. *atlantigena* St. Yves (2n=8x=56, $G_1G_1G_2G_2M_1M_1M_2M_2$ genomes) and with decaploid *F. arundinacea* var. *letourneuxiana* and *cirtensis* (2n=10x=70, $QQG_1G_1G_2G_2M_1M_1M_2M_2$ genomes). *Festuca mairei* St. Yves (2n=4x=28, $M_1M_1M_2M_2$ genomes) is dissimilar to other species in morphology. It has rough leaves, well-developed veins, and genes for high photosynthesis (Randall et al. 1985). *F. arundinacea* var. *glaucescens* and *F. mairei* are native to North Africa where they are sympatric with *F. arundinacea* var. *atlantigena* and *F. arundinacea* var. *letourneuxiana* and *cirtensis* (Malik and Thomas 1967).

The genus *Lolium* contains three diploid species (2n=2x=14) of most interest to tall fescue geneticists and breeders; the outcrossing perennial *L. perenne* and annual *L. multiflorum* Lam., and self-pollinating *L. temulentum* L. These species are closely related to *Festuca* and readily cross with *Festuca* species (Terrell 1966).

Interspecific and intergeneric crosses involving *Lolium* and *Festuca* could potentially combine the better palatability and forage quality of *Lolium* with the higher persistence and tolerance to stress environments found in tall fescue. Therefore, crosses between tall fescue and *Lolium* species have been made (Terrell 1966; Thomas and Humphreys 1991) and commercial cultivars derived, such as 'Kenhy' (Buckner et al. 1977) and 'Johnstone' (Buckner et al. 1983). Kenhy showed significantly higher palatability and digestibility, and lower acid-detergent-fiber, than the 'Kentucky 31' and 'Fawn' tall fescue cultivars (Buckner et al. 1979).

Communicated by D. R. Pring

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This paper is a contribution of the Missouri Agricultural Experimental Station, Journal Series no. 11,798

Table 1 Plant species and genotypes used. Letters in parenthesis indicate their genomic constitution

Genotype no.	Source of genotypes	2n no.	Species
1	303.1–1	14	*F. pratensis* Huds. (PP)
2	307.1–3	"	"
3	D1334-1	"	"
4	D1342-1	"	"
5	Stella-13	"	"
6	Bn354-2	28	*F. arundinacea* var. *glaucescens* Boiss. ($G_1G_1G_2G_2$)
7	Bn354-5	"	"
8	Bn574-2	"	"
9	Bn586-86–1	"	"
10	Bn586-86–2	"	
11	Mairei #1	28	*F. mairei* St. Yves ($M_1M_1M_2M_2$)
12	Mairei #2	"	"
13	Mairei #3	"	"
14	Kentucky 31	42	*F. arundinacea* var. *genuina* Schreb. ($PPG_1G_1G_2G_2$)
15	Martin	"	"
16	Mozark	"	"
17	MO-96	"	"
18	HYT	"	"
19	Bonanza	"	"
20	Rebel II	"	"
21	Bn275-6	56	*F. arundinacea* var. *atlantigena* St. Yves ($G_1G_1G_2G_2M_1M_1M_2M_2$)
22	Bn867-4	"	"
23	I20-15	"	"
24	PI283-283–6	"	"
25	Bn275-3	70	*F. arundinacea* var. *letourneuxiana* and *cirtensis* ($QQG_1G_1G_2G_2M_1M_1M_2M_2$)
26	I16-2		
27	PI283-283–5	"	
28	Derby	14	*L. perenne* L. (LL)
29	Gator	"	"
30	Regal	"	"
31	Manhattan	"	"
32	Linn	"	"

Restriction fragment length polymorphisms (RFLPs) have been used to study phylogenetic relationships of crop plants such as those found in the *Brassica* species (Song et al. 1988). Our previous study showed that the genetic relatedness among tall fescue genotypes could be estimated by RFLP analysis, and we identified genome-specific probes which hybridized only to one or two of the species used (*F. pratensis*, *F. arundinacea* var. *glaucescens*, and *F. arundinacea* var. *genuina*) (Xu et al. 1991). The tall fescue probes also cross-hybridized to *L. perenne* DNA and detected an average polymorphism of 69% (Xu et al. 1992).

The objectives of the present research were to evaluate genome specificity of a sample of DNA probes and to examine the relationships among *Festuca* and *Lolium* species based on RFLP data obtained with a common set of probes.

Materials and methods

Twenty-seven plants from six *Festuca* species and five from *L. perenne* were used (Table 1). Their ploidy levels were confirmed by counting chromosome numbers in root-tip samples. Genomic DNA extraction, gel electrophoresis, Southern blotting, and hybridization were as described previously (Xu et al. 1991). Genomic DNA was digested with the enzymes *Eco*RI or *Hin*dIII and 15 μg of digested DNA were loaded per lane. Three lanes of molecular-weight-markers were included in a 30-lane blot and two lanes for the 5-lane blots. Filters were washed in 2 x SSC plus 0.5% SDS and 0.1 × SSC plus 0.1% SDS at room temperature for 10 min each, and then in 0.1 x SSC and 0.1% SDS at 65 °C for 2 h.

Twenty-six single-copy and two repetitive cloned DNA probes were chosen from a *Pst*I-genomic library constructed from a hexaploid tall fescue plant (Xu et al. 1991). Plasmid DNA was isolated from transformed bacteria according to Birnboim (1983). Inserts were labelled to high specific activity with α-^{32}P dCTP based on the oligolabelling method of Feinberg and Vogelstein (1984).

The RFLP patterns on the autoradiograms were scored by considering each probe-enzyme combination as a locus. Each band was considered as a variant because the allelic nature of the bands was unknown. All bands were used in comparing genotypes, regardless of their frequency within a genotype. Genetic distances among all possible pairs of comparisons were estimated from Rogers' distance equation (Rogers 1972):

$$RD_{ij} = \frac{\sum_{k=1}^{L} \sqrt{\frac{1}{2}\sum_{n=1}^{A_k} (P_{ikn} - P_{jkn})^2}}{L}$$

where L is the number of RFLP 'loci', A_k is the number of RFLP variants at the kth RFLP 'locus', and P_{ikn} and P_{jkn} are the frequencies of the nth RFLP variant at kth RFLP locus in genotype i and j, respectively. The values of P_{ikn} and P_{jkn} were 1 when the band was present and 0 when the band was absent. Associations among genotypes were determined from cluster analysis on the basis of Rogers' genetic distances using the average cluster procedure of SAS (SAS 1985).

Table 2 *Pst*I-genomic clones used and their hybridization patterns[a]

DNA clone	Size (bp)	Hybridization to species[b]							Clone type
		LP	FP	FG	FM	FAG	FAA	FAL	
TF096	897	+	+	+	+	+	+	+	A
TF109	1259	+	+	+	+	+	+	+	A
TF124	420	+	+	+	+	+	+	+	A
TF144	261	+	+	+	+	+	+	+	A
TF165	897	+	+	+	+	+	+	+	A
TF171	420	+	+	+	+	+	+	+	A
TF209	815	+	+	+	+	+	+	+	A
TF213	333	+	+	+	+	+	+	+	A
TF265	815	+	+	+	+	+	+	+	A
TF411	870	+	+	+	+	+	+	+	A
TF412	1174	+	+	+	+	+	+	+	A
TF425	280	+	+	+	+	+	+	+	A
TF428	794	+	+	+	+	+	+	+	A
TF524	2167	+	+	+	+	+	+	+	A
TF531	1083	+	+	+	+	+	+	+	A
TF153	316	–	+	–	+	+	+	+	B
TF173	1312	–	–	–	+	+	+	+	C
TF212	1916	–	–	–	+	+	+	+	C
TF214	473	+	–	+	–	+	+	+	D
TF294	986	–	–	+	+	+	+	+	E
TF296	558	–	–	+	+	+	+	+	E
TF316	416	–	–	+	+	+	+	–	E
TF318	804	–	–	+	+	+	+	+	E
TF515	1403	–	–	+	+	+	+	+	E
TF544	1178	–	–	+	+	+	+	+	E
TF416	1380	–	–	–	–	+	+	+	F
TF513	1151	–	–	+	–	+	+	+	G
TF436(R)	1174	–	–	R	R	R	R	R	H
TF521(R)	1710	R	R	–	–	R	S?	S?	I

[a] +, with hybridization signal; –, no hybridization signal; R, hybridized as repetitive sequence; and S, hybridized as single-copy sequence

[b] LP, *Lolium perenne* (2x, L genome); FP, *F. pratensis* (2x, P genome); FG, *F. arundinacea* var. *glaucescens* (4x, G_1G_2 genomes); FM, *F. mairei* (4x, M_1M_2 genomes); FAG, *F. arundinacea* var. *genuina* (6x, PG_1G_2 genomes); FAA, *F. arundinacea* var. *atlantigena* (8x, $G_1G_2M_1M_2$ genomes); FAL, *F. arundinacea* var. *letourneuxiana* and *cirtensis* (10x, $QG_1G_2M_1M_2$ genomes)

Results and discussion

Different hybridizing patterns in various species

The 28 tall fescue *Pst*I-genomic probes showed different hybridizing patterns with DNA of the seven species and were thus classified into nine types (Table 2). Type-A probes hybridized with all species and included 15 in number. Type-B probes, represented by TF153, hybridized to five of the species but not to *L. perenne* and *F. arundinacea* var. *glaucescens*. Type-C probes, including TF173 and TF212, did not hybridize to the diploids or to the tetraploid *F. arundinacea* var. *glaucescens*. Type-D probes (TF214 typical) hybridized to the remaining species other than *F. pratensis* and *F. mairei*. Type-E probes hybridized to all polyploids. This type of probe included TF294, TF296, TF316, TF318, TF515, and TF544. Type-F probes (TF416 typical) hybridized to the hexaploids, octoploids, and decaploids but not to the tetraploids and diploids. Type-G probes (TF513 typical) hybridized to species, other than *F. mairei* and the diploids. Type-H probes (TF436 typical) behaved like Type-E except it was described as a repetitive sequence probe. Type-I probes (TF521 typical) are repetitive sequence probes that hybridized to all species except the tetraploids. Different hybridization patterns, especially at the diploid and tetraploid levels, may reflect the evolutionary relationships among these species.

The hybridization patterns were similar for *L. perenne* and *F. pratensis* (Table 2). Several probes did not hybridize to *L. perenne* and *F. pratensis*, but they did hybridize to higher-level polyploids. This suggested that the genomes in *L. perenne* and *F. pratensis* are very similar and relatively diverged from at least one of the genomes present in the higher-level polyploids.

The complexity of hybridization patterns varied with different types of probes and species. The higher-ploidy species showed more complicated banding patterns, especially with Type-A probes (Fig. 1). The average numbers of bands detected in individual plants of the seven species was significantly different (Table 3). The number of bands in the polyploid species was twice or more that of the diploid species. This is particularly apparent for the Type-A probes which hybridized to all seven species (Table 4). In comparison with non-specific probes, genome-specific probes showed simpler banding patterns and less variation among species (Fig. 1).

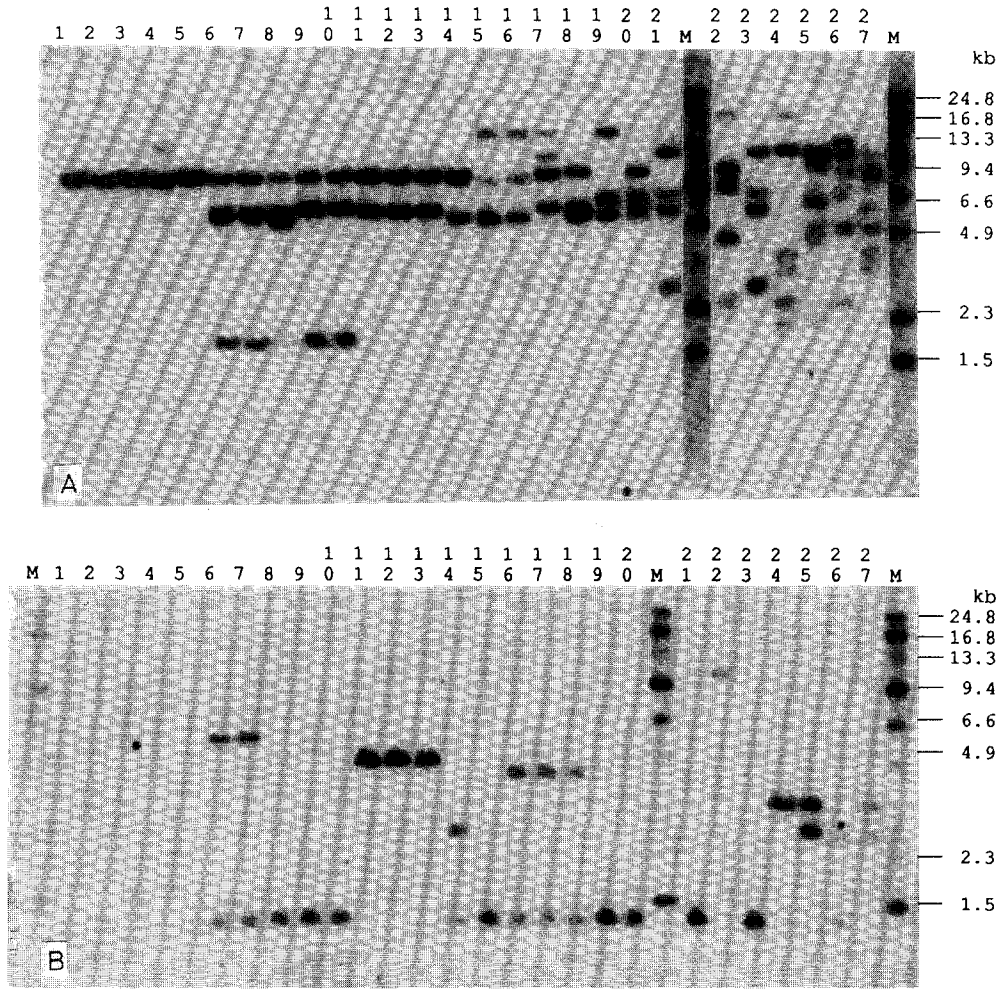

Fig. 1A, B Autoradiograms of *Hin*dIII-digested DNA from genotypes 1–27 probed with TF524 (**A**) and TF318 (**B**)

Table 3 Comparisons of the average number of bands detected in individual genotypes of various species

Species[a]	Ploidy	Average number of bands in a genotype		
		*Eco*RI	*Hin*dIII	Combined
LP	2X	1.01	1.04	1.02
FP	2X	0.81	0.91	0.86
FG	4X	1.82	1.97	1.90
FM	4X	2.17	2.36	2.26
FAG	6X	2.19	2.33	2.26
FAA	8X	2.35	2.86	2.61
FAL	10X	2.60	3.00	2.80
Mean		1.85	2.07	1.96
LSD (0.05)		0.64	0.71	0.47

[a] LP, *Lolium perenne* (2x, L genome); FP, *F. pratensis* (2x, P genome); FG, *F. arundinacea* var. *glaucescens* (4x, G_1G_2 genomes); FM, *F. mairei* (4x, M_1M_2 genomes); FAG, *F. arundinacea* var. *genuina* (6x, PG_1G_2 genomes); FAA, *F. arundinacea* var. *atlantigena* (8x, $G_1G_2M_1M_2$ genomes); FAL, *F. arundinacea* var. *letourneuxiana* and *cirtensis* (10x, $QG_1G_2M_1M_2$ genomes)

Table 4 Average number of bands detected in each species by different types of probes

Probe type	No. of probes	Enzymes[a]	Species[b]						
			LP	*FP*	*FG*	*FM*	*FAG*	*FAA*	*FAL*
A	15	E	1.67	1.33	2.07	2.53	2.80	3.00	3.47
		H	1.73	1.52	2.16	2.98	3.09	3.72	4.00
B	1	E	0.00	1.40	0.00	2.00	1.71	1.00	2.67
		H	0.00	1.80	0.00	1.00	1.57	1.20	2.67
C	2	E	0.00	0.00	0.00	1.50	1.50	1.25	0.84
		H	0.00	0.00	0.00	1.00	1.95	1.25	0.65
D	1	E	1.40	0.00	2.00	0.00	2.29	2.25	0.33
		H	2.00	0.00	3.20	0.00	2.43	3.00	2.00
E	6	E	0.00	0.00	2.36	2.60	2.76	2.00	2.13
		H	0.00	0.00	2.77	2.67	2.70	2.42	2.56
F	1	E	0.00	0.00	0.00	0.00	2.29	0.50	0.67
		H	0.00	0.00	0.00	0.00	1.00	1.00	0.00
G	1	E	0.00	0.00	2.20	0.00	0.14	0.75	0.00
		H	0.00	0.00	1.00	0.00	0.43	0.25	0.33

[a] E, *Eco*RI; H, *Hin*dIII

[b] LP, *Lolium perenne* (2x, L genome); FP, *F. pratensis* (2x, P genome); FG, *F. arundinacea* var. *glaucescens* (4x, G_1G_2 genomes); FM, *F. mairei* (4x, M_1M_2 genomes); FAG, *F. arundinacea* var. *genuina* (6x, PG_1G_2 genomes); FAA, *F. arundinacea* var. *atlantigena* (8x, $G_1G_2M_1M_2$ genomes); FAL, *F. arundinacea* var. *letourneuxiana* and *cirtensis* (10x, $QG_1G_2M_1M_2$ genomes)

DNA markers would help alien gene introgression

Probes showing differential hybridization patterns (±) to various species are useful molecular markers for genetic and breeding studies in the *Festuca-Lolium* complex. One use of these probes would be to verify interspecific and intergeneric hybrids, and further to monitor gene introgression between these species. For example, Type-B and Type-D probes (such as TF153 and TF214) can verify F_1 hybrids between *L. perenne* and *F. pratensis*. These DNA markers will enhance the use of wide crosses in tall fescue improvement.

Repetitive sequences TF436 and TF521

In this study, TF436 hybridized only to the polyploid species. TF521 hybridized to diploids, hexaploids, octoploids and the decaploid, but not to the two tetraploid species. More studies are necessary on their distribution within the genomes. Eucaryotic genomes contain a high proportion of repetitive sequences (Flavell 1980). Repetitive-sequence probes isolated from rye (*Secale cereale* L.) have been used as chromosomal markers for the identification of alien chromosomes (or chromosome segments) present in wheat (*Triticum aestivum* L.) (Appels and Moran 1984). Genomic-specific repetitive DNA sequences have also been cloned and characterized in other plants such as rice (*Oryza* spp.) (Aswidinnoor et al. 1991). These sequences may be useful in monitoring alien gene introgression between species.

Clustering analysis of RFLP data

The average Rogers' genetic distances between all pairs of genotypes [n=32, total pair number is n(n-1)/2=496] were calculated using RFLP data from 53 probe-enzyme combinations.

Except for the octoploid and decaploid genotypes (Fig. 2), all genotypes within a species were clearly clustered in a single group. The results showed that the genetic distance between *F. pratensis* and *L. perenne* was the lowest, suggesting that the relatedness between these two diploids was highest. The *F. pratensis* and *L. perenne* group was further clustered with *F. arundinacea* var. *glaucescens* (tetraploid), then with *F. arundinacea* var. *genuina* (hexaploid), forming a larger subgroup. This subgroup then clustered with *F. mairei* (tetraploid) and the octoploid and decaploid genotypes. When individuals were clustered in the same group, their relationships were assumed to be closer. *Festuca mairei* was less similar to these four species, suggesting that it was more distinct from tall fescue. The high association between *F. pratensis* and *L. perenne* may be because these diploids have undergone considerable introgression. Perhaps the misplacement of the octoploid and decaploid genotypes was because their high ploidy levels led to difficulty in the discrimination of bands.

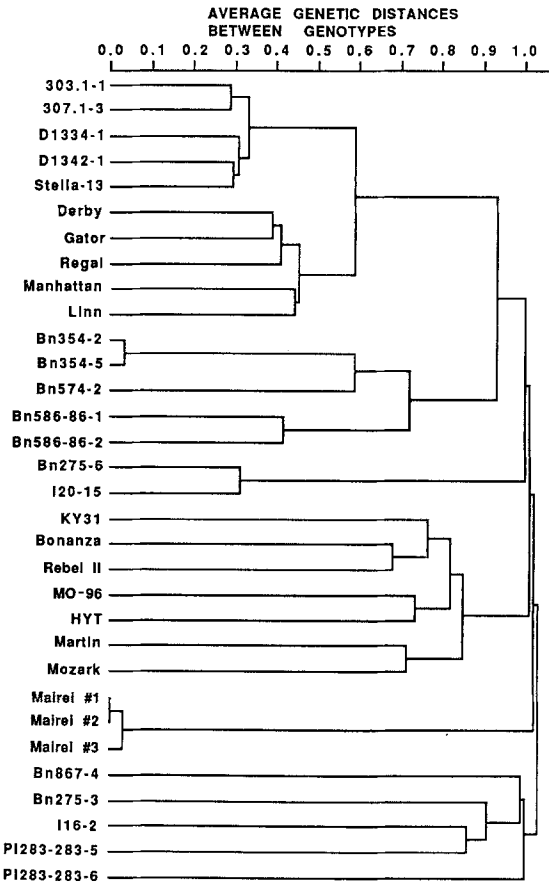

Fig. 2 Associations of the genotypes and species of *Festuca* and *Lolium*

Associations among genotypes produced by cluster analysis largely agreed with the known pedigree information. In the *F. arundinacea* var. *glaucescens* group, for example, genotypes Bn354-2 and Bn354-5, and Bn586-86–1 and Bn586-86–2 are half-sibs and they formed a sub-group. In the seven hexaploid tall fescue groups, 'Bonanza' and 'Rebel II' are turf-type tall fescues and, as a result, the two were clustered in a sub-group. Does this reflect genetic differences between turf and forage cultivars? Further studies are needed with more genotypes to determine this. 'Martin' and 'Mozark' are two cultivars developed at the University of Missouri and the plants from these two cultivars were clustered together.

In conclusion, the differential (±) hybridization patterns of 29 tall fescue *Pst*I DNA probes indicated genome divergence in six *Festuca* species and *L. perenne*. The genome-specific probes will be useful molecular markers to verify hybrids and to monitor gene introgression for interspecific and intergeneric hybridization. The dendrogram generated by cluster analysis reflected the phylogenetic relationships among these species.

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