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# A genetic linkage map of tef [*Eragrostis tef* (Zucc.) Trotter] based on amplified fragment length polymorphism

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**Abstract** A genetic linkage map of tef was constructed with amplified fragment length polymorphism (AFLP) markers using $F_5$ recombinant inbred lines (RILs) derived by single seed descent from the intraspecific cross of 'Kaye Murri' × 'Fesho'. A total of 192 *Eco*RI/*Mse*I primer combinations were screened for parental polymorphism. Around three polymorphic fragments per primer combination were detected, indicating a low polymorphism level in tef. Fifty primer combinations were selected to assay the mapping population, and 226 loci segregated among 85 $F_5$ RILs. Most AFLP loci behaved as dominant markers (presence or absence of a band), but about 15% of the loci were codominant. Significant deviations from the expected Mendelian segregation ratio were observed for 26 loci. The genetic linkage map comprised 211 markers assembled into 25 linkage groups and covered 2,149 cM of genome. AFLP is an efficient marker system for mapping plant species with low polymorphism such as tef. This is the first genetic linkage map constructed for tef. It will facilitate the mapping of genes controlling agronomically important traits and cultivar improvement in tef.

**Key words** Tef · *Eragrostis tef* · Genetic linkage map · AFLP

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## Introduction

Tef is one of the major cereal crops in Ethiopia (Ketema 1993), and Ethiopia is the center of origin and diversity of tef (Vavilov 1951). It is a small-grained (2–3 mg per kernel), sexual, autogamous, allotetraploid, C4-metabolism cereal plant of medium stature and short growth duration (Moffett and Hurcombe 1949; Tavasoli 1986; Jones et al. 1978; Berhe et al. 1989; Ayele et al. 1996). It has 40 chromosomes (2n = 4x = 40) with a relatively small genome size of about 730 mega basepairs (Jones et al. 1978; Ayele et al. 1996). Tef contains high levels of proteins with an excellent balance among the essential amino acids, and the flour is also rich in mineral content, especially in iron, zinc, copper, and manganese (Ketema 1993). It is adapted to a wide range of climatic and soil conditions, especially water-stress environments, and is also suitable for long-term storage without pest damage (Tefera et al. 1990). Despite the importance of tef as cereal crop, information on its molecular genetics is lacking. It is widely recognized that the construction of a linkage map in many species greatly increases the efficiency of genetic improvement. Thus, the development of a molecular linkage map in tef will enhance our understanding of tef genetics and improve the efficiency of crop breeding, especially when quantitative traits are involved.

Several types of DNA markers have been successfully used for genetic mapping in many species. Restriction fragment length polymorphisms (RFLPs), a hybridization-based assay system, have been a valuable source of markers for the construction of molecular maps in plants and enable synteny studies because of their locus specificity (Tanksley et al. 1992; Ahn and Tanksley 1993; Causse et al. 1994; Kesseli et al. 1994; Sherman et al. 1995). However, the laborious procedure involved and the few loci detected per assay limit its application for high-resolution mapping and marker–assisted selection in a breeding program. Random amplified polymorphic DNAs (RAPDs), a polymerase chain reaction (PCR)-based assay, allows a relatively large number of genetic loci to be assayed rapidly

and inexpensively (Williams et al. 1990). This system has been used to analyze genetic relationships within and between species (Ratnaparkhe et al. 1995; Millan et al. 1996) and to construct genetic maps (Kesseli et al. 1994; Sonder et al. 1996). However, the sensitivity of RAPD markers to subtle changes in reaction conditions is the major limitation in a large-scale application of RAPD in genome research. Amplified fragment length polymorphism (AFLP) is a recently developed multilocus polymorphic marker technique which combines the strengths of different marker systems and provides a new opportunity for mapping in plants with large genomes and low polymorphism (Vos et al. 1995). AFLP assays can detect a larger number of genetic loci per reaction than RFLP or RAPD analysis in many crops (Becker et al. 1995; Mackill et al. 1996; Schondelmaier et al. 1996). As our preliminary results revealed low genetic diversity in tef using RFLPs and RAPDs (unpublished data), a molecular marker with the capacity to assay large numbers of loci with minimal effort is especially important for genome mapping. The construction of a genetic linkage map of tef would establish a basis for the identification and genetic manipulation of agronomically important quantitative traits. In the investigation presented in this paper, we employed AFLP markers to construct the first genetic linkage map of tef.

## Materials and methods

### Plant materials

The map was generated from AFLP analysis of $F_5$ recombinant inbred lines (RILs) of an intraspecific cross between the tef cultivars 'Kaye Murri' and 'Fesho'. $F_2$ seeds available from previous studies (Tefera 1993) of the two cultivars were used to develop $F_2$-derived $F_5$ RILs using single-seed descent. Cultivars 'Kaye Murri' and 'Fesho' manifest extreme phenotypic characteristics for several qualitative and quantitative traits (Tefera 1993; Tefera and Peat 1997a, b). 'Kaye Murri' is a tall, thick-culmed, late-maturing, white-seeded cultivar with red lemmas and very compact panicles. By contrast, 'Fesho' is a short, thin-culmed, early-maturing, brown-seeded cultivar with purple lemmas and very loose panicles. The $F_2$ seeds were initially germinated in petri dishes; 400 individual plants were subsequently transplanted to 12–cm pots in May 1995 and grown to maturity in the glasshouse at the Debre Zeit Agricultural Research Center, Ethiopia. Maturity in tef on a panicle basis progresses from top to bottom and, therefore, all of the spikelets do not mature at the same time. As a result, one spikelet (3–8 kernels) from the top portion of the main panicle of individual plants was detached when the pedicel dried up and germinated immediately in petri dishes to establish an $F_3$ population. Two seedlings from each $F_2$ plant were transplanted to 12–cm pots; later on, 1 seedling was removed, leaving the other to grow to maturity. The $F_4$ generation was also raised in a similar way; at maturity, each $F_4$ plant was harvested and threshed. $F_5$ seeds from a random sample of 85 $F_4$ plants/lines were germinated indoors under light conditions, and then each seedling was transplanted to a 7.5-l pot maintained in the greenhouse at Texas Tech University, Lubbock, Texas in May 1996. The plants were watered as required and fertilized once a week with Miracle-Gro.

### DNA preparation

Leaf tissue from each $F_5$ RILs was excised 3 weeks after transplanting and stored in a freezer at –70°C. DNA was isolated using the method described by McCouch et al. (1988). The DNA was dissolved in distilled sterile water. The final DNA concentration was determined by Fluorometer (Hoefer Scientific Instruments, Model TKO–100, San Francisco, Calif.) according to the manufacturer's instructions. Approximately 500 ng of genomic DNA was completely double–digested with 5 U each of *Mse*I and *Eco*RI endonucleases at 37°C for 3 h. The digested DNA fragments were ligated with *Eco*RI and *Mse*I adapters (Table 1) for 3 h at 20°C. Ligated DNA template was diluted to ten times and stored at –20°C.

**Table 1** Adapters and primers used for pre–amplification and selective amplification

| Name of primer/adapter: | Sequence (5'–3') |
|---|---|
| **1) Adapter:** | |
| *Eco*RI adapter | CTCGTAGACTGCGTACC<br>CTGACGCATGGTTAA |
| *Mse*I adapter | GACGATGAGTCCTGAG<br>TACTCAGGACTCAT |
| **2) Primer:** | |
| ***Eco*RI primer:** | |
| E–[a] | GACTGCGTACCAATTC |
| E–AA | GACTGCGTACCAATTCAA |
| E–AAC | GACTGCGTACCAATTCAAC |
| E–AAG | GACTGCGTACCAATTCAAG |
| E–ACA | GACTGCGTACCAATTCACA |
| E–ACC | GACTGCGTACCAATTCACC |
| E–ACG | GACTGCGTACCAATTCACG |
| E–ACT | GACTGCGTACCAATTCACT |
| E–AG | GACTGCGTACCAATTCAG |
| E–AGC | GACTGCGTACCAATTCAGC |
| E–AGG | GACTGCGTACCAATTCAGG |
| E–AGT | GACTGCGTACCAATTCAGT |
| E–CG | GACTGCGTACCAATTCCG |
| E–GC | GACTGCGTACCAATTCGC |
| E–TG | GACTGCGTACCAATTCTG |
| E–CA | GACTGCGTACCAATTCCA |
| E–CT | GACTGCGTACCAATTCCT |
| ***Mse*I primer:** | |
| M–[b] | GATGAGTCCTGAGTAA |
| M–ACC | GATGAGTCCTGAGTAAACC |
| M–AGC | GATGAGTCCTGAGTAAAGC |
| M–CAA | GATGAGTCCTGAGTAACAA |
| M–CAC | GATGAGTCCTGAGTAACAC |
| M–CAG | GATGAGTCCTGAGTAACAG |
| M–CAT | GATGAGTCCTGAGTAACAT |
| M–CT | GATGAGTCCTGAGTAACT |
| M-CTA | GATGAGTCCTGAGTAACTA |
| M–CTC | GATGAGTCCTGAGTAACTC |
| M–CTG | GATGAGTCCTGAGTAACTG |
| M–CTT | GATGAGTCCTGAGTAACTT |
| M–GCG | GATGAGTCCTGAGTAAGCG |

E–[a] and M–[b] refer to *Eco*RI and *Mse*I primers without selective nucleotides, respectively. Primers E– and M– were directly used for the pre-amplification of template DNA. The AFLP fingerprint was generated using pairs of *Eco*RI and *Mse*I primers with two or three selective nucleotides as listed above. All possible primer combinations (192 combinations) between *Eco*RI and *Mse*I primers were used to screen parents for AFLPs. Only 50 primer combinations were selected for further evaluation of $F_5$ progeny

### PCR amplification

The AFLP protocol developed by Keygene (Vos et al. 1995) was used with minor modifications. All primers and adapters were synthesized by Operon Technologies (Alameda, Calif.). Two consecutive amplifications with primers containing none, two, or

three selective nucleotide extensions (Table 1) were carried out in a PE–9600 thermocycler (Perkin Elmer Corp., Norwalk, Conn.). For preamplification, a 40-µl PCR reaction mix contained 10 µl of resuspended ligated DNA template, 70 ng of each of the two primers (*Eco*RI and *Mse*I) without selective nucleotides, 1× reaction buffer (10 m*M* TRIS–HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$), 1 U of *Taq* polymerase, and 250 µ*M* of each dNTP. The following cycle profile was used for PCR amplification: a 30-s DNA denaturation step at 94°C, a 1-min annealing step at 56°C, and a 1-min extension step at 72°C, for 30 cycles. After thermal cycling, the PCR products were diluted 30–fold in TE buffer and used as a DNA template for further selective amplification. For selective amplification, 3.5 ng of *Eco*RI primer with two or three selective nucleotide extensions (Table 1) was labeled by phosphorylating the 5' end of the *Eco*RI primers using [$^{33}$P]-γATP. Labeling was carried out at 37°C for 1 h in a thermocycler, and the enzyme $T_4$ polynucleotide kinase was inactivated by heating at 70°C for 10 min. Twenty nanograms of *Mse*I primer was added and mixed with 250 µ*M* of dNTPs, 1×PCR buffer, 0.5 U of *Taq* polymerase, and 3 µl of the pre-amplified and diluted DNA template, in a final volume of 12 µl. The selective amplification (36 cycles) was performed by programming a thermocycler with a regime of denaturation for 30 s at 94°C, annealing for 30 s at 65°C, followed by an extension reaction of 1 min at 72°C for 1 cycle. For the subsequent 11 cycles, the annealing temperature was lowered by 0.7°C per cycle. For the final 24 cycles, the denaturation step was carried out for 30 s at 94°C, annealing for 30 s at 56°C, and extension for 1 min at 72°C.

### Gel analysis

Following amplification, reaction products were mixed with an equal volume (12 µl) of formamide dye (98% formamide, 100 m*M* EDTA, pH 8.0, and bromophenol blue, and xylene cyanol which served as a tracking dye). The resulting mixture was heated for 5 min at 94°C, and then quickly cooled on ice. Each sample (3 µl) was loaded onto a 5% denaturing polyacrylamide gel. The gel matrix was prepared with 4.75% acrylamide, 0.25% methylene bisacryl-amide, and 7.5 *M* urea in 0.5 × TBE buffer (50 mM TRIS, 50 m*M* boric acid, 1 m*M* EDTA). To 100 ml of gel solution, 600 µl of 20% ammonium persulfate and 75 µl TEMED was added right before the gel was cast with a SequiGen 38 × 50-cm gel apparatus (BioRad, Hercules, Calif.). Two hours after casting, the gel was pre-run in 0.5× TBE buffer for more than 0.5 h at 100 W constant wattage before the samples were loaded. After electrophoresis, the gel was dried on a standard slab gel drier (BioRad, Hercules, Calif.) for 2 h and then exposed to X-ray film for 1–4 days depending upon the intensity of the radiation signal.

### Map construction

Amplified fragments were visually scored as present or absent and thereby assigned to either parental allele for map construction. To ensure accurate scoring, we scored all AFLP markers at least twice. Ambiguous genotypes were resolved by assigning a blank score (–) to the individual locus for map construction. Linkage analysis was performed using MAPMAKER version 2.0 (Lander et al. 1987) on a Power Macintosh computer. Linkage groups were established using the 'group' command with LOD ≥3 and maximum recombination frequency of 0.4. The order of loci within each linkage group was determined by the 'three point' and 'first order' commands, then verified by the 'ripple' command. The 'try' command was used to find possible linkages with unassigned loci and small linkage groups. Map distances were calculated using the Kosambi mapping function (Kosambi 1944). A chi–square test was performed to determine if the allele frequency at each individual locus deviated from the expected 1:1 segregation ratio for the recombinant inbred population (Steel and Torrie 1980).

## Results and discussion

### Polymorphism and inheritance of AFLP markers in tef

Screening of the two parental lines ('Kaye Murri' and 'Fesho') with 192 primer pairs gave 25–125 (average of 65) scorable fragments per reaction. The average polymorphism level between the parental lines was 4%. Based on the results of the parental assay, the RILs were evaluated with 50 selected primer combinations. These primers produced 3,675 fragments, of which 226 were polymorphic (6.1%). The level of polymorphism varied with the primer combination and ranged from 1% to 17.5%. The polymorphism level detected in tef is lower than that observed in other self-pollinated crops like rice (Mackill *et al.* 1996) and sugar beet (Schondelmaier *et al*. 1996) where 28% and 50% polymorphism was reported, respectively. Low polymorphism (11%) has also been reported in barley (Becker *et al.* 1995). Our previous diversity analysis of 47 tef cultivars using RAPDs and AFLPs also showed a similar result (unpublished data). Tef can, therefore, be considered as one of the crop species having low levels of DNA polymorphism. Possible reasons for this are: (1) tef has an extremly low level of out crossing (Kedir 1991), and (2) the cultivation of tef is restricted to a particular geographical area (primarily grown in Ethiopia).

The number of selective nucleotides of the *Eco*RI primer significantly affected the number of amplified AFLP fragments. A similar comparison was made for *Mse*I primers with three selective nucleotides. *Eco*RI primers with two and three selective nucleotides amplified an average of 92.8 and 55.7 fragments per reaction, respectively. In general, *Eco*RI primers with two selective nucleotides amplified more polymorphic fragments (5 polymorphic fragments per reaction on average) than primers with three selective nucleotides (4.1 polymorphic fragments per reaction on average). However, a relatively larger number of faint bands were observed in the amplification with two selective nucleotides than with three selective nucleotides.

An example of AFLP segregation patterns in the $F_5$ is shown in Fig. 1. A great majority of the AFLP markers followed Mendelian segregation in the $F_5$ RILs, indicating that these AFLP markers behave as heritable traits. However, 26 markers (11.5%) significantly deviated from a 1:1 Mendelian segregation ($P \leq 0.05$). The polymorphic fragments were derived almost equally from both parents (124 from 'Fesho' and 136 from 'Kaye Murri'). AFLPs were mainly scored as dominant markers (85%) in tef, but some showed codominant segregation which is more than that reported in barley and rice (Maheswaran et al. 1997; Becker et al. 1995). Most codominant amplification fragments in tef were very close to each other in their molecular weight, as observed in rice (Maheswaran et al. 1997). A codominant marker is generally considered to be the most informative because it can identify heterozygous locus. The codominant

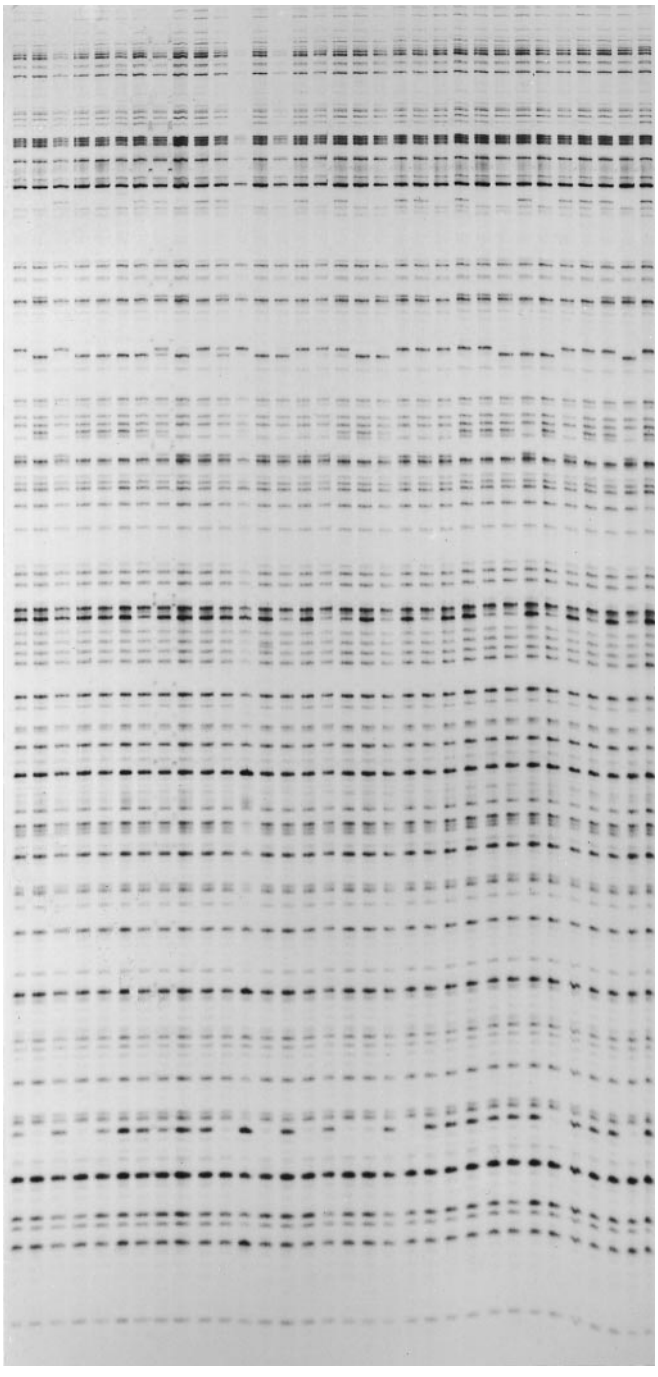

**Fig 1** AFLP segregation pattern in $F_5$ recombinant inbred lines (RILs) from the cross of 'Kaye Murri' × 'Fesho'. The fingerprint was amplified by the primer pair E–CA/M–AGC. The leftmast *two lanes* are produced by amplifying DNA template from parental lines 'Fesho' and 'Kaye Murri', respectively. *Lanes 3–32* were amplified using template DNA from a random sample of the $F_5$ RILs

markers identified in this study might be valuable for gene mapping and marker-assisted selection in early generation evaluation.

## AFLP linkage analysis

A total of 226 polymorphic bands were scored by analyzing RILs of tef. Linkage groups and frameworks were established with the MAPMAKER program. Loci with distorted segregation were also included in the analysis. The genetic linkage map comprised 211 markers assembled into 25 linkage groups with at least 3 markers in each group at a LOD ≥3 (Fig. 2). Of the 226 loci tested, 15 remained unlinked. The linked loci covered 2,149 cM and linkage groups varied in size from 15.3 to197.4 cM. Genetic distances between markers ranged from 0 to 43.4 cM (on linkage group 16) with an average spacing of 10.4 cM. The number of mapped markers for each linkage group varied from 3 to 15. A high correlation ($r$ = 0.845; $P \leq 0.05$) was found between the length of the linkage groups and number of markers per linkage group, suggesting that AFLP markers are randomly distributed over the entire genome of tef. This has also been reported in barley (Becker et al. 1995) and rice (Maheswaran et al. 1997). On the other hand, a clustered distribution of AFLP markers was found in sugar beet (Schondelmaier et al. 1996).

Since the gametic chromosome number for tef is 20 (Jones et al. 1978), it is conceivable that we have not identified linkage groups corresponding to each chromosome. To saturate the map, we can add AFLP markers by screening more primer combinations of *Eco*RI/*Mse*I and by assaying more enzyme combinations other than *Eco*Rl/*Mse*I. Some stable RAPD markers may also be added to the present map. Since RFLPs allow synteny studies (Becker et al. 1995), an ongoing project at Texas Tech University is screening probes to add some RFLP markers onto the map for further comparative mapping. With the addition of more markers, the smaller linkage groups may converge or join with other linkage groups. Such a saturated map could be directly used for gene and quantitative trait locus (QTL) tagging. On the other hand, an interspecific cross may provide an alternative solution for low polymorphism. In the genus *Eragrostis*, about 350 species are available (Watson and Dallwitz 1992), and DNA polymorphism between tef and some of these species is very high (unpublished data). High polymorphism between tef and its relatives indicate that interspecific hybridization may enrich genetic resources for tef improvement and provide ideal mapping populations for the construction of a basic map and for the genetic study of agronomic traits. However, only *E. pilosa* is crossable with tef in the attempts made so far.

## Application of AFLP in tef improvement

Low polymorphism in tef creates a barrier for the application of molecular marker technologies in tef improvement. Much more effort has to be made to achieve the same level of progress in using marker technology as in other crops. However, the large number of fragments that can be generated by AFLP in one reaction (Vos et al.

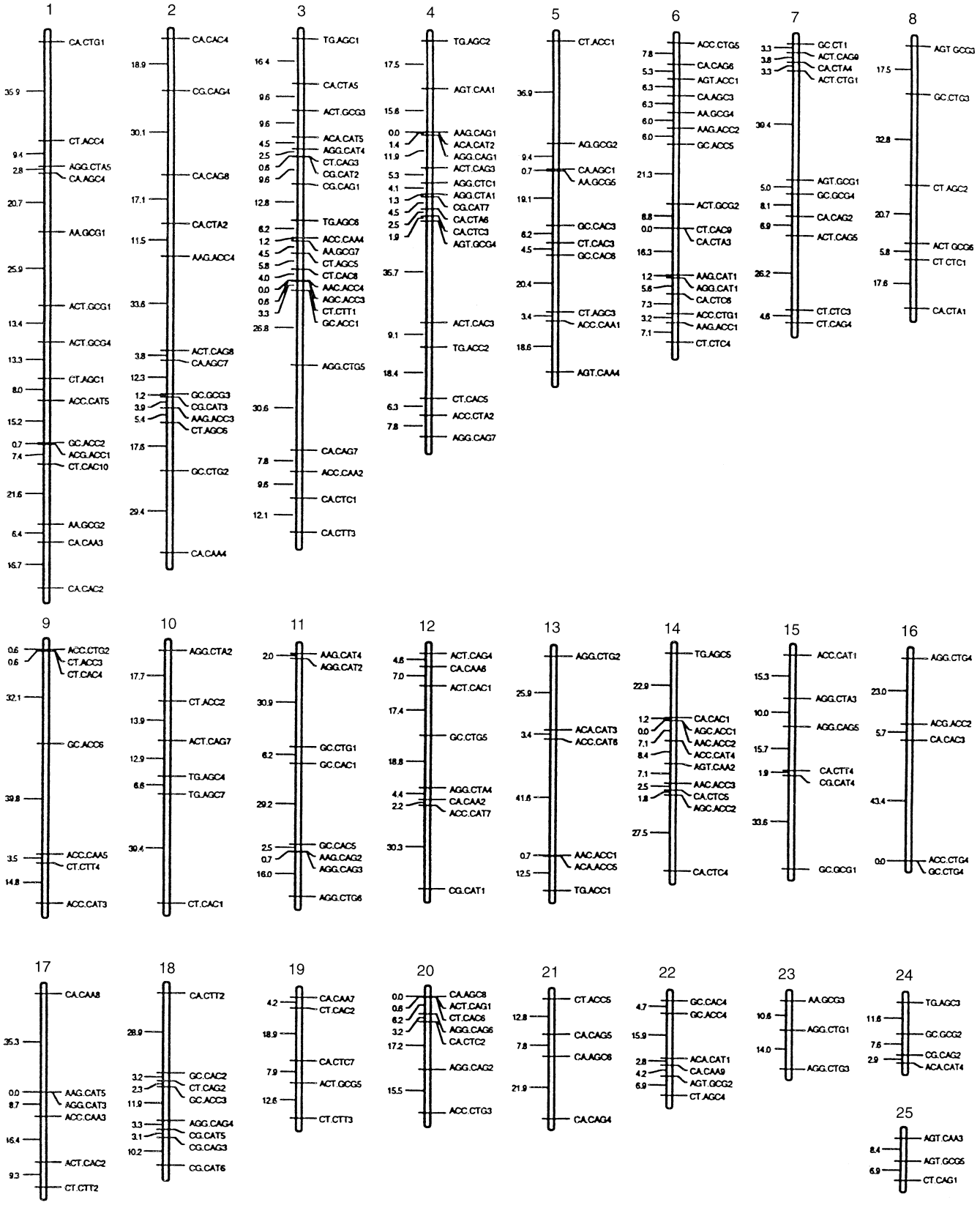


**Fig. 2** AFLP linkage map of *E. tef*. Linkage groups were denoted *1–25* in decreasing order of size. Map distances, in Kosambi centiMorgans, are indicated to the *left* of the linkage groups, and locus names [combination of primer names (*E* for *Eco*RI and *M* for *Mse*I) plus abbreviation of selective nucleotide extension in 3′-end of each primer] are given to the *right*.

1995; Thomas et al. 1995) increases the number of polymorphic fragments despite the low polymorphism of tef. In this study, 226 polymorphic fragments segregating in the mapping population were identified by one person in 5 months despite the low polymorphism in tef. In a preliminary study, only 20% of the random primers used in RAPD could detect polymorphism between the two parents studied (data not shown). In contrast, AFLP generated at least 1 polymorphic fragment between the parents with any primer combinations screened. With 50 selected primer combinations, an average of 4.5 informative fragments per primer pair were produced. AFLP may not offer the highest rate of polymorphism in terms of the ratio of polymorphic bands to total amplified bands (Becker et al. 1995), but it allows the simultaneous analysis of a large number of DNA fragments in one gel, which makes the technique more efficient. Because of its high reliability, high speed in generating polymorphism, and unlimited number of primer combinations available, AFLP is a powerful molecular tool to study tef genome.

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