November 1992

ENDOPHYTES OF TURFGRASSES: NEW TOOLS AND APPROACHES

Executive Summary

This project was proposed and initiated by Dr. Peter Day, AgBiotech Center, and Dr. Reed Funk, Department of Crop Science, Rutgers. Our program goals are: (a) to produce a germplasm collection of fungal endophyte-infected grasses concentrating on *Poa* and *Agrostis* species; (b) to produce a collection of unifungal endophyte cultures for classical and molecular analysis; (c) to extend the host range of endophytes to grasses where they have not been found; (d) to produce endophyte-specific DNA probes; (e) to use the probes to characterize endophyte variability and produce RFLP maps for taxonomy; (f) to develop turfgrass tissue culture regeneration and transformation systems for the incorporation of agronomically important genes into turfgrass.

After extensive screening of collections of *Poa* and *Agrostis* species throughout the U.S.A. and Europe (in collaboration with Dr. Jim White, Auburn University, Alabama), we have obtained a limited number of fungal endophyte-infected grasses in these two genera. However, as of now we did not find fungal endophytes in Kentucky bluegrass (*Poa pratensis*) and Creeping bentgrass (*Agrostis palustris*). Therefore, we are currently developing an *in vitro* culture system for extending the host range of endophyte to Kentucky bluegrass and Creeping bentgrass. To date, a collection of unifungal endophyte cultures has been established and contains representative isolates from a wide variety of turfgrass genera. We are using mycelial macerates of these endophyte isolates to inoculate embryogenic callus lines of *Poa pratensis* and *Agrostis palustris* to obtain novel combinations of endophyte infected grasses. We have inoculated several embryogenic callus lines of creeping bentgrass with endophytes and successfully regenerated plants. Regenerating grasses were planted in the soil and are currently being examined for the presence of endophyte in the leaf bases. Several are positive. We produced fungal endophyte-specific DNA probes by the polymerase chain reaction (PCR) and diagnostic fingerprints of DNA sequences generated by randomly amplified polymorphic DNA (RAPD) PCR method. RAPD markers obtained with four ten base random DNA primers were successfully used to discriminate among individual endophyte isolates. A phylogenetic tree of endophyte isolates was generated using RAPD markers produced from 5 random primers. We have also developed tissue culture and regeneration systems for Kentucky bluegrass and Creeping bentgrass. The embryogenic callus lines established provide alternative tissues for creating endophyte containing grasses and target cells for the development of a stable transformation system by particle bombardment. Embryogenic callus lines with high regeneration potentials have been established with several varieties of Kentucky bluegrass and Creeping bentgrass. We are using *E. coli* β-glucuronidase as a reporter gene to assay transient expression of an added gene in grass cells after bombardment. Various parameters were optimized to improve transient expression in cultured bentgrass to high levels. We are currently selecting for stably transformed turfgrass cell lines which express a *bar* gene conferring resistance to the herbicide bialaphos (Basta™). For identification of putative transformants, we used a well known tissue culture model system, Black Mexican Sweetcorn (BMS) suspension cells, to obtain BMS clones resistant to bialaphos through particle bombardment and with these clones, we developed a PCR assay for early identification of transgenic turfgrass plants. Regenerants from putative transformed callus will be confirmed by Southern blot hybridization and phosphinothricin acetyltransferase (PAT) assay. Other single gene traits of interest confer insect resistance, virus resistance, and resistance to fungal and bacterial pathogens.

I. Turfgrass germplasm and endophyte culture collection

A. Endophyte culture

During 1990, Dr. Jane Breen completed the initial phase of extensive screening of turfgrass germplasm collections, especially *Poa* and *Agrostis* species throughout the U.S. and Europe (in collaboration with Dr. Jim White, Auburn University, Alabama). We did not find the presence of fungal endophytes in Kentucky bluegrass and Creeping bentgrass.

Our unifungal endophyte cultures have increased from 24 isolates (collected and cultured by Dr. Breen, 1990) to 30 isolates with cultures obtained from Dr. White (1991) and Dr. Funk (1992). These isolates were collected from a variety of turfgrass genera (Table 1) including several *Agrostis* species such as *A. alba, A. hiemalis, A. stolonifera, A. tenuis,* and several *Poa* species such as *P. palustris, P. ampla, P. mo,* and *Festuca rubra* var. *commutata* (chewing fescue) and *Festuca ovina* var. *duriuscula* (hard fescue).

B. Endophyte inoculation to create novel endophyte/turfgrass combinations

To test our hypothesis that embryogenic callus lines with high regeneration potentials may provide a new route for endophyte infection, we did the preliminary tests using mycelial macerates of AS4 (endophyte isolate associated with choke-forming creeping bentgrass in England) to inoculate embryogenic callus lines of three creeping bentgrass varieties, Putter, Emerald and Southshore. After inoculations, the embryogenic calluses were regenerated through our regeneration procedure. The numbers of regenerants recovered (Table 2) were similar to the numbers of regenerants from controls (Table 7).

Putter and Emerald regenerants were rooted, transferred to soil, hardened, and were growing well in greenhouse conditions. Half of the regenerants (about 100 plants) were planted in the field with the help of Dr. Dave Huff (Crop Sciences) to collect information on survival of tissue culture regenerants in the field. Seeds from these plants will be harvested next summer. Presence of endophyte in these plants will be further tested. Initial microscopic examinations for the presence of endophyte hyphae in the leaf bases with the other half of regenerants showed that a small number of plants might have endophytes. Further examinations with these plants at different developmental stages are necessary to confirm the presence of endophytes. Regenerants from Southshore bentgrass have also been transferred to the greenhouse and will be examined later for the presence of endophytes.

The success of producing regenerants in the presence of endophyte inoculum provides a basis for testing many endophyte isolates against several embryogenic callus lines. We will test the feasibility of inoculation with combined multiple isolates and will then proceed with large-scale testing inoculation. We would like to conclude in the future that the high regeneration potential of our embryogenic callus lines provide a new route for infection with the appropriate endophyte isolates.

II. DNA Fingerprinting

A. Endophyte specific DNA probes

As we mentioned in the report of November 1991, in collaboration with Dr. Christopher Schardl (University of Kentucky, Plant Pathology Department), we seek to obtain more precise DNA sequence information to aid fungal endophyte taxonomy by obtaining the DNA sequences of the poorly conserved spacer regions between the more highly conserved ribosomal RNA genes. Thus, Intergenic Transcribed Spacer (ITS-1) sequences between the 5.8S ribosomal RNA gene and the 18S ribosomal RNA gene were amplified from genomic DNAs of endophyte isolates by the polymerase chain reaction (PCR). The ITS-1 DNA fragments from *Acremonium starrii* isolates from Longfellow's chewing fescue (LF2) and PP10 (*Poa palustris* inoculated), and *Acremonium typhinum* isolate 1210-1, were inserted into a plasmid vector PCR™1000 using a commercial "TA cloning™" system. These clones can be used as endophyte specific probes.

B. Identification of endophyte isolates with RAPD markers

Endophyte DNAs were isolated from fungal mycelia following the procedure of Lee & Taylor(1). DNA yields varied from 2 to 40 µg per gram fresh weight of mycelium. Even the lowest yield provides enough DNA to run hundreds of Randomly Amplified Polymorphic DNA (RAPD)-Polymerase Chain Reaction (PCR). RAPD-PCR uses a single random primer, normally a ten-mer, to produce various lengths of DNA fragments that can be treated as markers for identification(2).

As mentioned in the May 1992 report, we continued RAPD-PCR screening of all of our endophyte isolates. Several primers (Table 4) were used to amplify fungal genomic DNAs using the slightly modified PCR protocol from Williams et al.(3). An example of RAPD products generated by primer A07 is shown in Fig. 1. For *Acremonium* endophyte isolate identification, seven RAPD markers selected from four primers provided sufficient differences to define a unique pattern for each isolate (Table 5). Twenty seven RAPD markers from 5 primers (A03, A04, A05, A07, A08) were used in a cluster analysis (SYSTAT program) to reveal relatedness among the endophyte isolates. The tree diagram (Fig. 2) showed several groups with *A. chilense* most distant from the rest of the isolates and *A. coenophialum* closely related to the Blue 3 isolate *A. typhinum,* some *A. starrii* isolates were more closely related to *A. typhinum* isolates than to other *A. starrii* isolates.

We will use RAPD-PCR with unidentified or unknown endophyte isolate DNAs to test the application of RAPD markers for discriminating among endophyte isolates from nature and from new grass/endophyte combinations. We also expect to recover endophyte specific probes from among the amplified DNAs that can be used to detect endophytes in host tissue.

III. Turfgrass tissue culture and regeneration and transformation

A. Embryogenic callus initiation and regeneration

Callus initiation from mature seeds and selection of embryogenic callus lines were periodically started with several varieties of Kentucky bluegrass (*Poa pratensis*) and Creeping bentgrass (*Agrostis palustris*) to establish a number of embryogenic callus lines as target tissues for endophyte inoculation to produce endophyte containing grasses and for transformation studies. Embryogenic callus lines were selected from all varieties of Creeping bentgrass tested (Table 6). These embryogenic callus lines were regenerated and the lines with high regeneration potential were used to initiate embryogenic suspension cultures. Table 7 showed regeneration percentages of some embryogenic callus lines. Most lines had high regeneration rates.

B. Transformation

As reported in November 1991, we have begun transforming grass with foreign DNA using microprojectile bombardment. Both embryogenic callus and embryogenic suspension cells were used as targets. The *E. coli* β-*glucuronidase* (GUS) gene was used as a scorable marker and the *bar* gene which confers resistance to the herbicide bialaphos, was used as a selectable marker. Using the transient assay of GUS expression in turfgrass tissues and a standard tissue culture system BMS (Black Mexican Sweetcorn suspension cells), we are optimizing the particle bombardment protocol for stable transformation. Table 8 summarizes the particle bombardment experiments to date. We tested many variables to optimize the bombardment parameters and selection conditions. As reported in May 1992, we found that colloidal gold particles were less toxic to Putter bentgrass cultured cells than the conventional tungsten particles although both gold and tungsten particles work equally well for BMS cells. Other bentgrass cultured cells such as Emerald and Southshore also responded better to gold than tungsten particles. Pretreatment of grass cultured cells with 0.4M mannitol containing maintenance medium also increased transient expression of the GUS constructs.

We have obtained BMS clones resistant to bialaphos and used them to establish a PCR assay to confirm the presence of the *bar* coding region in extracted DNA. This has been used to screen turfgrass colonies growing on selection media. As shown in Table 8, six selection experiments are in progress and PCR screening will be used to identify putative turfgrass transformants with the *bar* gene. We will regenerate plants from these colonies. Phosphinothricin acetyltransferase (PAT) assay and southern blot hybridization will be used for additional confirmation.

IV. Related Projects

A. A complementary project, "Screening for extrachromosomal genetic elements in *Acremonium* endophytes," was funded by the Rutgers Turf Research Program. A Ph.D. student, Chan-Seok Oh who was partly supervised by Dr. T.M.A. Wilson, continued to screen *Acremonium* isolates for presence of extrachromosomal elements. He has screened all isolates in our collection without finding any. As reported in May 1992, a reliable method for isolating and regenerating protoplasts of the fungus has been developed. Results from antibiotics screening showed that *Acremonium* isolates are sensitive to hygromycin B.

Research continued on the different extrachromosomal elements previously identified in high choke (virulent) and low choke (less virulent) isolates of *Atkinsonella hypoxylon*. A cDNA library representing dsRNAs in high choke *A. hypoxylon* was synthesized and mapped. Clones representing the 2.5 and 2.1 kbp dsRNAs identified in this strain have been almost completely sequenced, and coding regions identified. No similarity with other dsRNAs known to affect fungal virulence has been detected. Efforts to cure this strain of extrachromosomal elements by single sporing revealed that these elements appear to be transmitted through 100% of single conidia.

B. Another project, "Development of enhanced herbicide resistance and abiotic stress tolerance through transformation" was funded at $154,109 for two years by the Rutgers Center for Interdisciplinary Studies in Turfgrass Science. This project involves collaboration with Dr. Chee-kok Chin and Dr. Gojko Jelenkovic (Dept. of Horticulture) and Dr. Barbara Zilinskas (Dept. of Biochemistry & Microbiology). Dr. Christina Hartman, a postdoctoral research associate funded by the project is currently working with Dr. Lisa Lee.

C. A third project, "Development of herbicide resistant turfgrass through mutant selection and transformation" will be funded at $47,430 for one year by the New Jersey Commission on Science and Technology.

V. Future Directions

Dr. Nilgun Tumer began supervision of the laboratory work of the project in October 1992.

We will continue to work on (1) use of RAPD markers for isolate identification, (2) introduction of endophytes into turfgrass using the tissue culture system, and (3) transformation of turfgrass with agronomically important genes.

VI. Presentations and Meetings (1992). Dr. Lee attended the following meetings.

1. 1992 World Congress on Cell and Tissue Culture, 20-25 June 1992

2. 32nd Grass Breeders Work Planning Conference, 16-18 August 1992 -- presentation

3. Rutgers Turfgrass Research Field Day, 6 August 1992

4. Second Annual Turfgrass Symposium, January 1993 -- presentation

VII. References

1. Lee, B. Steven and John W. Taylor. (1990) Isolation of DNA from fungal mycelia and single spores. In PCR Protocols: A Guide to Methods and Applications. pp. 282-287. Eds. M.A. Innis, D.H. Gelford, J.J. Sninsky, and T.J. White. Academic Press: San Diego.

2. Hu, Jinguo and Carlos F. Quiros. (1991) Identification of broccoli and cauliflower cultivars with RAPD markers. Plant Cell Reports 10: 505-511.

3. Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski, and S.V. Tingey. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531-6535.

Table 1. Collection of Endophytes in Culture

Endophyte	Host Species	Source notation
Acremonium chilense		
A. coenophialum	*Triumph*	
A. huerfanum	*Festuca arizonica*	
A. starrii	*Agrostis alba* (red top)	Type III, Pluckemin, NJ
	Festuca rubra var. *commutata* (chewing fescue)	LF2 LF3 LF4
	Festuca ovina var. *duriuscula* (hard fescue)	ST1 ST3 ST4
	Poa palustris	PP8 PP10 PP17 PP Halstead
A. typhinum	*Agrostis hiemalis*	[A3] Type II, Montgomery, AL
	Agrostis stolonifera	[AS4] Type I, England
	Agrostis tenuis	[AT1] Type I, England [AT3] Type I, England
	Dactylis glomerata	
	Festuca rubra	213-3 Icelandic 231 (low resistance) 245-1 Icelandic 245 (high resistance)
	Festuca rubra	blue 2 blue 3 blue 4 1209-7 50% choke 1210-1 No choke 1211-5 50% choke
	Poa ampla *Poa mo*	*Ampla 90*
Atkinsonella hypoxylon	*Danthonia spicata*	1-low choke 2-high choke

Table 2. Preliminary tests of endophyte inoculations to create new endophyte/turfgrass combinations

	Endophyte	Embryogenic callus	Total numbers of regenerants
Exp. 1	AS4	PB.1 (30 pieces)	275
Exp. 2	AS4	EB.5 (50 pieces)	380
Exp. 3	AS4	SSB.1 (10 pieces)	10 (1 piece)

Table 3. DNA yields of *Acremonium* endophyte isolates

Isolate	FW(g)	Yield (ug/g)
chilense	1.25	15.8
coenophialum	0.45	42.5
alba	0.56	15.7
LF3	0.83	6.5
ST1	0.71	7.6
ST3	0.81	6.2
ST4	0.71	14.3
PP8	0.92	38.5
PP10	0.81	20.0
PP17	0.80	2.0
PPH	1.11	15.6
AS4	0.56	5.7
AT1	0.47	28.1
Ice245-1	0.57	10.6
Blue2	1.64	5.9
Blue3	0.67	14.4
Blue4	0.60	18.0
1210-1	2.50	2.3
1211-5	1.16	4.7
Ampla 90	0.66	40.1
Poa MO	0.39	43.6

Table 4. DNA sequences of RAPD primers

A-03	AGTCAGCCAC
A-04	AATCGGGCTG
A-05	AGGGGTCTTG
A-07	GAAACGGGTG
A-08	GTGACGTAGG
A-09	GGGTAACGCC
A-10	CAGCACCCAC

Table 5. RAPD markers for Acremonium isolate identification

	Isolate	A-03 430	A-03 1500	A-04 470	A-04 700	A-04 1400	A-05 1200	A-07 370	A-07 1800
1	A. chilense	-	-	+	-	-	+	-	+
2	A. coenophialum	-	+	-	+	+	-	-	-
	A. starrii								
4	LF3	+	-	-	+	-	+	+	-
6	ST3	+	-	+	-	-	-	+	-
7	ST4	+	+	-	-	-	-	+	-
5	ST1	-	-	-	-	-	+	+	-
8	PP8	-	+	+	+	+	-	-	-
8	PP10	-	+	+	+	+	-	-	-
9	PP17	+	-	+	+	-	-	-	-
10	PPH	+	+	+	+	+	-	-	-
3	alba	+	-	-	+	+	-	-	-
	A. typhinum								
12	AT1	-	-	-	+	+	-	-	-
11	AS4	-	-	-	+	-	-	-	+
16	1210-1	+	-	+	-	-	+	+	-
17	1211-5	+	-	+	+	-	+	+	+
13	Blue2	+	-	+	+	-	-	+	-
15	Blue4	+	-	-	-	-	-	+	-
14	Blue3	+	-	-	+	+	+	-	-

Table 6. List of Creeping Bentgrass Varieties

Putter bentgrass
Emerald bentgrass
Penncross
Southshore
SR1020
Providence

Table 7. Regeneration of some embryogenic callus lines

Line	Plates	Total Colonies	% Regeneration	Ave. # of Shoots
Putter 1	3	56	98	7.3
Putter 3	2	44	36	1.3
Putter 4	5	96	87	4.1
Emerald 2	4	83	8	0.3
Emerald 4	1	20	90	8.9
Emerald 5	5	105	90	4.9
Emerald 6	2	32	0	0

Table 8. Summary of Particle Bombardment Experiments Using Embryogenic Cell Suspensions

Exp.#	# of Shot	Colloidal Particle type	Transient Expression	Stable Transformation
1	8	M10*	very low	-
2	16	M10	very low	-
3	20	M10	very low	-
4	28	M10	ok	NA**
5	30	M10 & Gold	ok, gold > m10	-
6	14	M10 & Gold	good, gold > m10	NA
7	24	Gold	low	plants in greenhouse
8	19	M10 & Gold	low	-
9	13	M10 & Gold	low	-
10	14	M10 & Gold	low	in selection
11	24	M10	low	NA
12	24	Gold	good	in selection
13	20	Gold	ok	in selection
14	18	Gold	good	in selection
15	22	Gold	very low ***	in selection
16	28	Gold	good	in selection

* M10: tungsten particles

** no selectable marker used

*** embryogenic callus lines of turfgrass were used as target tissue.

marker 1 2 3 4 5 6 7 8 8 9 10 11 12 13 14 15 16 17 marker

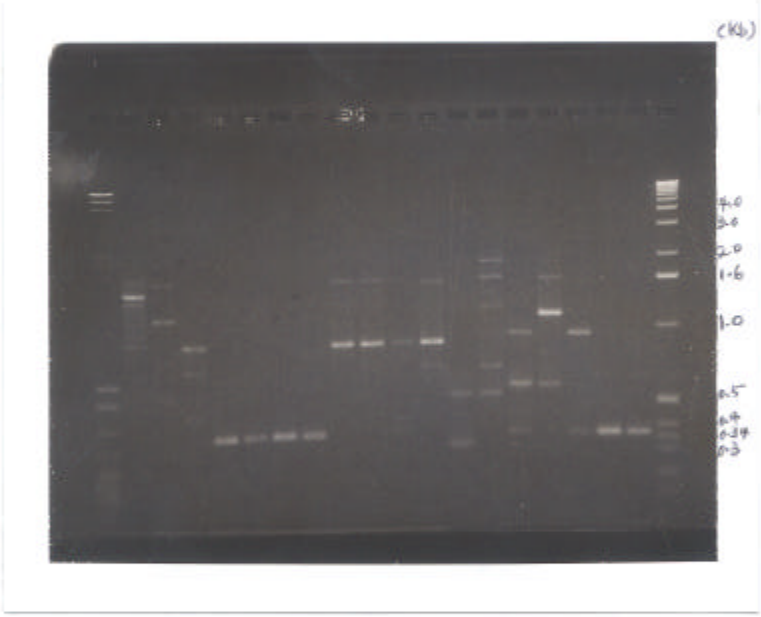

Figure 1. RAPD products generated by primer AO7. The numbers on top refer to *Acremonium* endophyte isolates listed in Table 5.

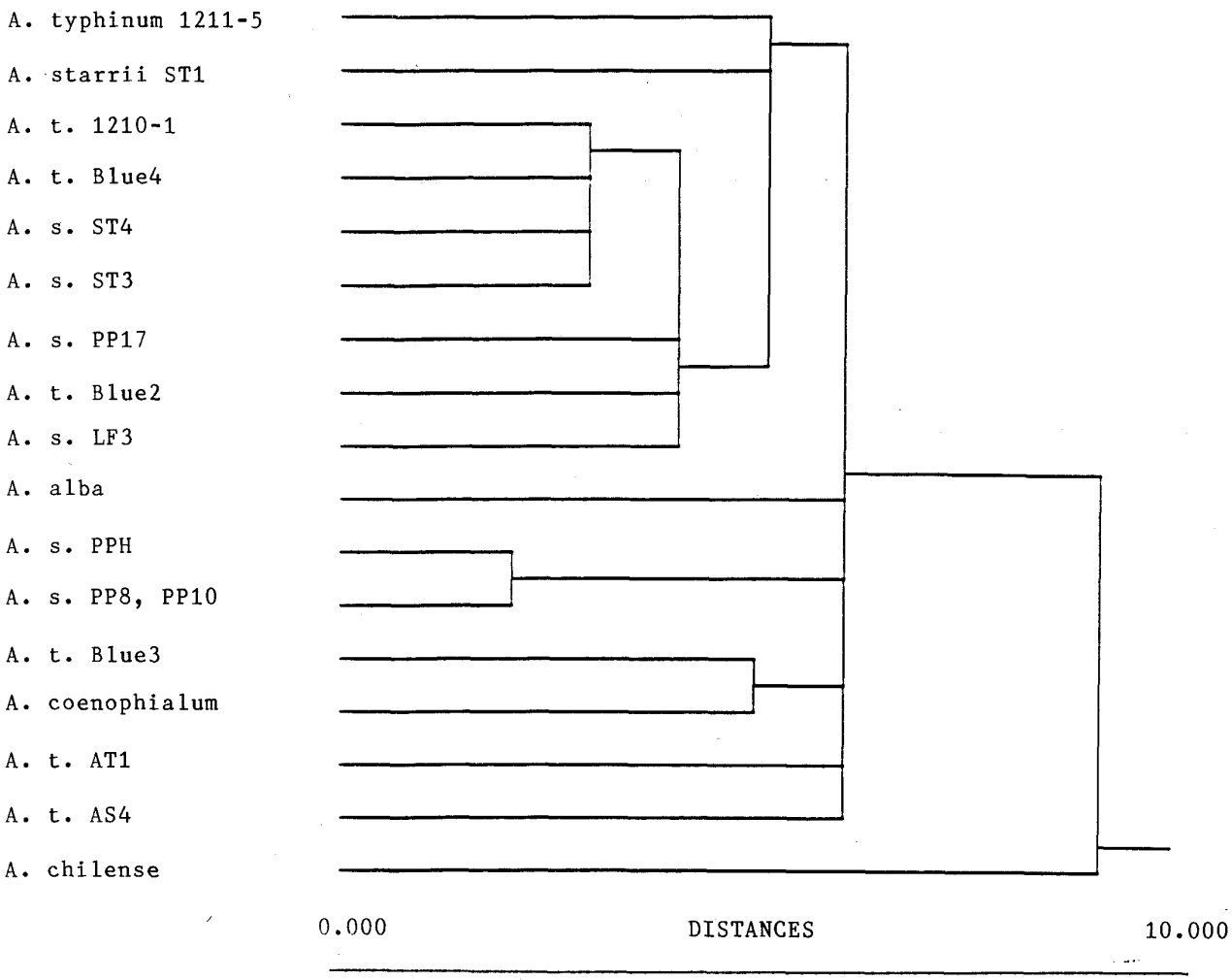

Fig. 2. Phylogenetic relationships of Acremonium endophyte isolates using RAPD markers generated from 5 random primers.