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Identification of Shiitake Genotypes by Multilocus Enzyme Electrophoresis: Catalog of Lines

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Starch gel electrophoresis of allozymes extracted from mycelium grown in submerged culture was used to identify and catalog lines of Lentinula edodes. *Variability at 11 multiallelic loci was used to separate 91 lines collected from worldwide sources into 35 genotypic clases. Genotypic class 2 was the largest, with 18 lines; 18 other genotypic classes contained more than one line; and 16 genotypic classes contained only one line each. Three hybrids, with potential commercial value, were produced by crossing single-spore-derived monokaryons of different genotypes. Each of the three hybrids could be differentiated from the above 35 classes based on their multilocus allelic combinations. Multilocus enzyme analysis should aid the description, registration, and licensing of improved shiitake cultivars.*

KEY WORDS: fungi; shiitake; *Lentinula edodes;* genetic variation; allozymes; electrophoresis.

INTRODUCTION

Identification of lines of shiitake [*Lentinula edodes* (Berk.) Pegler (=*Lentinus edodes* (Berk.) Sing.)] is important for genetic improvement programs where large numbers of isolates must be evaluated for production characteristics. Such evaluation programs are limited in the number of isolates tested due to space, time, and labor constraints. In addition, the process of collecting

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germplasm to begin a breeding and selection program often results in the addition of duplicate germplasm to the collection because germplasm of common ancestry obtained from different sources almost always is labeled differently. A consistent system of cultivar naming and identification used for most crop plants is not used by the mushroom industry. The reasons for lack of a naming system are due, in part, to the absence of an unambiguous system of genotype identification. Due to the influence of environmental factors on morphological characteristics, morphology cannot readily be used to separate unique genotypes.

The one-to-one relationship of allozyme phenotype to genotype recently has been applied to genetic studies of fungi (Speith, 1975; May *et al.*, 1979a; Royse and May, 1982a, b; Royse *et al.*, 1983a; Tooley *et al.*, 1985; Shattock *et al.*, 1986). Most early electrophoretic studies of fungi (Shechter *et al.*, 1973) were limited to scoring the presence or absence of general protein bands, with little regard for inheritance of discrete loci coding for specific enzymes. The latter approach takes advantage of the simple Mendelian genetic basis of allozyme variation and provides far more useful, genetically interpretable data.

In a previous study of *L. edodes,* we were able to separate 45 lines of *L. edodes* into 24 genotypic classes, based on 11 multiallelic loci (Royse *et al.*, 1983a). We found that, in many instances, lines within the same genotypic class could be traced to a common source. This work was important in the separation of available lines into genotypic classes and effectively halved the number of trials necessary to evaluate and catalog available germplasm (Diehle and Royse, 1986).

In this study, we expanded on our earlier work by cataloging 46 additional isolates of *L. edodes* obtained from Korea, Japan, Papua New Guinea, Taiwan, and the Philippines.

MATERIALS AND METHODS

Isolates. Ninety-one isolates of *L. edodes* were obtained from commercial sources or from natural substrates in the wild. Isolates were assigned a number and deposited in The Pennsylvania State University Mushroom Culture Collection (PSUMCC). Isolate numbers, isolate sources, and identification of sources are presented in Table I. All lines are maintained on potato–dextrose–yeast extract agar (PDYA) slants at 4°C (Jodon and Royse, 1979) or in liquid nitrogen or both.

Crosses. Single-spore-derived cultures, used for breeding stocks, were obtained as outlined by Royse *et al.* (1983b). Homokaryoses of the single-spore-derived stocks were confirmed by the absence of clamp connections on hyphae growing on PDYA. Crosses were attempted between these single-

Table I. Accession Numbers and Sources of Isolates of *Lentinula edodes* Deposited in The Pennsylvania State University Mushroom Culture Collection (PSUMCC)

PSUMCC Accession no.	Source
30	Japan, 1975 (L. C. Schisler)
60	Japan, 1975 (L. C. Schisler)
61	Japan, 1975 (L. C. Schisler)
72	Japan, 1975 (L. C. Schisler)
131	Japan, 1975 (L. C. Schisler)
268	Japan, 1975 (L. C. Schisler)
291	ATCC[a] 28759, 1980
292	ATCC 28760, 1980
293	ATCC 38222, 1980
294	ATCC 24352, 1980
295	ATCC 24462, 1980
296	Kinoko kit, 1981 (H. Mee)
297	Royal Mushroom, B-3, 1981 (S. Wong)
298	ATCC 38221
304	Royal Mushroom, KC-4 Donko, 1981 (S. Wong)
305	Royal Mushroom, KA-5, 1981 (S. Wong)
318	Japan, Koshin 18, 1981 (F. Howard)
320	Japan, K 230, 1981 (F. Howard)
321	Japan, Koshin 22, 1981 (F. Howard)
322	Japan, K 250, 1981 (F. Howard)
323	Japan, Donko, 1981 (F. Howard)
324	Ogasware, California, 1981
325	Gourmet Mushrooms, 1981 (M. Clark)
369	USFS,[b] RA-3-2E, 1982 (G. Leatham)
375	Mushroompeople, 82 (Donko), 1982 (B. Harris)
376	Mushroompeople, 82a (Koshin), 1982 (B. Harris)
377	Mushroompeople, 92a, 1982 (B. Harris)
378	Mushroompeople, 806, 1982 (B. Harris)
379	Royal Mushrooms, S45, 1982 (S. Wong)
380	Royal Mushrooms, S71, 1982 (S. Wong)
381	Royal Mushrooms, S1121, 1982 (S. Wong)
382	USFS, 904S, 1982 (G. Leatham)
383	USFS, 904M, 1982 (G. Leatham)
384	USFS, S-5 (10), 1982 (G. Leatham)
385	USFS, S-7 (1), 1982 (G. Leatham)
386	USFS, S-14, (1), 1982 (G. Leatham)
387	USFS, AC-1C-1D-2(3)-1, 1982 (G. Leatham)
388	USFS, AC-1C-1C-1C-1, 1982 (G. Leatham)
389	USFS, 1303M, 1982 (G. Leatham)
390	USFS, 1303AY, 1982 (G. Leatham)
391	USFS, PRL-2747, 1982 (G. Leatham)
392	USFS, 117 = 1 + C, 1982 (G. Leatham)
393	Kinoko, 1982 (H. Mee)
394	W-4, 1982 (R. Kurtzman)
400	Taiwan, 1982, (D. Royse)
451	MRIJ,[c] 1985 (K. Mori)
452	MRIJ, 1985 (K. Mori)
453	MRIJ, 1985 (K. Mori)
463	Japan, Mori M121, 1985 (B. Harris)

Table I. (*Continued*)

PSUMCC Accession no.	Source
464	Elmer Schmidtt (Le2), 1985 (B. Harris)
468	Green Empire, Sy-1, 1985 (S. Youn)
469	Japan, Mori 465, 1985 (B. Harris)
470	Japan, Mori 505, 1985 (B. Harris)
471	Japan, Mori 701, 1985 (B. Harris)
472	Japan, Mori 290, 1985 (B. Harris)
473	Japan, Mori 252, 1985 (B. Harris)
474	Japan, Mori 440, 1985 (B. Harris)
487	Japan, Tokyo Market, 1985 (B. Harris)
488	Japan, Tokyo Market, 1985 (B. Harris)
489	Japan, Tokyo Market, 1985 (B. Harris)
490	Japan, Tokyo Market, 1985 (B. Harris)
542	IAS,[d] Korea, 1985 (Y. H. Park)
543	IAS, Korea, 1985 (Y. H. Park)
544	IAS, Korea, 1985 (Y. H. Park)
545	IAS, Korea, 1985 (Y. H. Park)
563	IAS, Korea, 1986 (Y. H. Park)
564	IAS, Korea, 1986 (Y. H. Park)
565	IAS, Korea, 1986 (Y. H. Park)
585	IAS, Korea, 1986 (Y. H. Park)
587	IAS, Korea, 1986 (Y. H. Park)
588	IAS, Korea, 1986 (Y. H. Park)
589	IAS, Korea, 1986 (Y. H. Park)
590	IAS, Korea, 1986 (Y. H. Park)
591	IAS, Korea, 1986 (Y. H. Park)
592	IAS, Korea, 1986 (Y. H. Park)
593	IAS, Korea, 1986 (Y. H. Park)
603	HRIO[e] No. 32, 1986 (D. L. Rinker)
604	IAS, Korea, 1986 (Y. H. Park)
641	IAS, Korea, 1986 (Y. H. Park)
642	IAS, Korea, 1986 (Y. H. Park)
643	IAS, Korea, 1986 (Y. H. Park)
645	IAS, Korea, 1986 (Y. H. Park)
647	IAS, Korea, 1986 (Y. H. Park)
648	IAS, Korea, 1986 (Y. H. Park)
649	IAS, Korea, 1986 (Y. H. Park)
650	IAS, Korea, 1986 (Y. H. Park)
651	IAS, Korea, 1986 (Y. H. Park)
652	IAS, Korea, 1986 (Y. H. Park)
653	IAS, Korea, 1986 (Y. H. Park)
666	Philippines, 1986 (T. H. Quimio)
675	IAS, Korea, 1986 (Y. H. Park)

[a] American Type Culture Collection, Rockville, Maryland.
[b] United States Forest Service, Madison, Wisconsin.
[c] Mushroom Research Institute of Japan, Kiryu, Japan.
[d] Institute of Agricultural Sciences, Suweon, Korea.
[e] Horticultural Research Institute of Ontario, Ontario, Canada.

spore-derived homokaryons in dual culture on PDYA. Two selected lines were allowed to grow toward each other on PDYA contained in 9-cm petri dishes. Mycelial plugs from the interaction zone were first transferred to agar to determine the presence or absence of mycelial clamp connections. Plugs, of mycelium observed with clamp connections, were than transferred to sterilized rye grain contained in 500-ml flasks. Fruit bodies were produced on synthetic logs as outlined by Royse (1985). A tissue culture of the fruit body was made on PDYA, then transferred to potato–dextrose–yeast extract broth (PDYB) to test for heterozygosity at any of the 11 loci.

Starch Gel Electrophoresis. Mycelial mats growing in PDYB (Royse *et al.*, 1983a) were vacuum-dried on Whatman No. 1 filter paper and transferred to 1.2-ml liquid nitrogen (Nunc) tubes. Three to five drops of extraction buffer (Tris–HCl, 0.05 M, *p*H 7.1) were added to the samples (about 0.1 g of vacuum-dried mycelium per sample) prior to freezing in a vapor-phase liquid nitrogen freezer until used (up to 1 month). At the time of enzyme assay,

Table II. Enzymes, Abbreviations, EC Numbers, Loci, Alleles with Mobility Designations, and Buffer Systems Used in this Study

Enzyme	Abbr.	EC No.	Loci	Alleles[a]	Buffer
Aspartate aminotransferase	AAT	2.6.1.1	Aat-1	1 = 100, 2 = 69, 3 = 110	C[b]
Diaphorase	DIA	1.6.4.3	Dia	1 = 100, 2 = 105	R[c]
Glutamate dehydrogenase (NADP+)	GDH	1.4.1.4	Gdh	1 = 100, 2 = 105, 3 = 95, 4 = 89	R
Glucosephosphate isomerase	GPI	5.3.1.9	Gpi	1 = 141, 2 = 100	C
Malate dehydrogenase	MDH	1.1.1.37	Mdh-1	1 = 100, 2 = 171, 3 = 203, 4 = 0	C
			Mdh-2	1 = 100, 2 = 115	
Mannosephosphate isomerase	MPI	5.3.1.8	Mpi	1 = 100, 2 = 89, 6 = 0	M[d]
Peptidase		3.4.3.1			
With leucyl-leucyl-leucine	PEP-LLL		Pep-1-LLL	1 = 100, 2 = 81, 6 = 0	M
With glycyl-leucine	PEP-GL		Pep-2-GL	1 = 100, 2 = 97	
Phosphoglycerate kinase	PGK	2.7.2.3	Pgk	1 = 50, 2 = 100, 3 = 150, 4 = 75	C
Superoxide dismutase	SOD	1.15.1.1	Sod	1 = 100, 2 = 50, 3 = 133	M

[a]Relative mobilities are presented for each allele, where the homomeric protein product of each allele is assigned a value relative to 100.
[b]Electrode buffer: 0.04 M citric acid adjusted to *p*H 6.1 with *N*-(3-aminopropyl)morpholine, diluted 1:10 for gel buffer, <90 mA (Clayton and Tretiak, 1972).
[c]From Ridgeway *et al.* (1970); 250 V (<75 mA).
[d]From Markert and Faulhaber (1965); 275 V (<75 mA), electrode buffer diluted 1:3 for gel.

samples were allowed to thaw at room temperature, then transferred immediately to 12 × 75-mm disposable glass test tubes. The samples were ground with a glass rod and centrifuged at 1000 *g* for 5 min. Samples were plated on 14% starch gels according to the methods of May *et al.* (1979b). Staining procedures were those of Allendorf *et al.* (1977), Royse *et al.* (1983a), and Shaw and Prasad (1970). The system of genic nomenclature of Allendorf *et al.* (1977) and May *et al.* (1979b) was used.

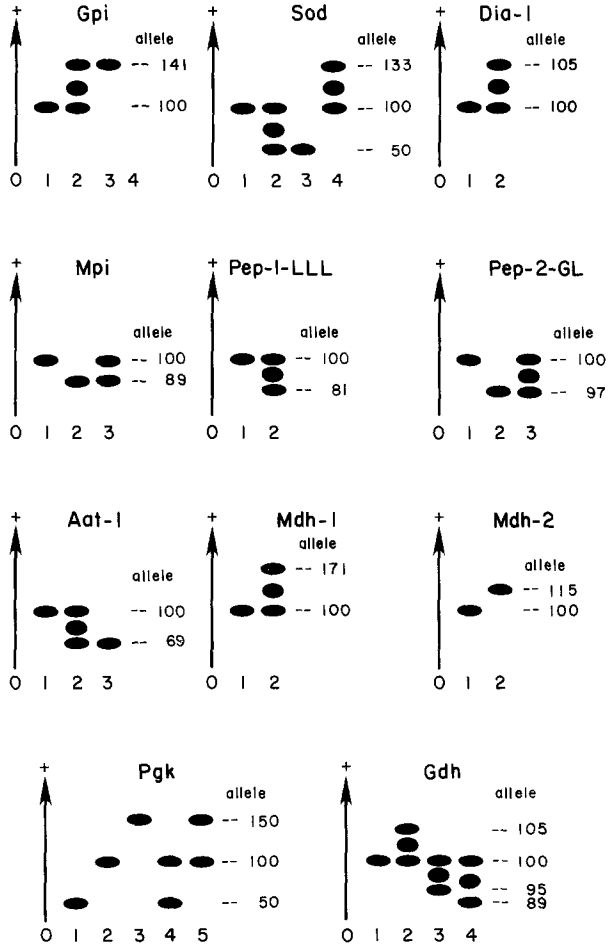

Fig. 1. Electrophoretic phenotypes with interpretable genetic bases observed for 11 biochemical marker loci used to identify genotypic classes of *Lentinula edodes*. For example, phenotype 1 for GPI has the genotype 100/100, while phenotype 2 for GPI has the genotype 100/141. Homomeric protein products are designated by the allele that codes for each.

Genetic similarities (Rogers, 1972) and cluster analysis by the UPGMA method (Sneath and Sokal, 1973) were calculated with a Fortran computer program ("Allozyme") written by R. Strauss (University of Arizona).

RESULTS

Nine enzymes, coded by 11 loci, were used in this study to catalog isolates of *L. edodes*. Table II presents these enzymes, their abbreviations, EC numbers, loci, and alleles with mobility designations, and the best buffer for the enzyme's resolution. Interpretation of genetic bases was made in accordance with known subunit compositions of these enzymes (Darnall and Klotz, 1972) and in accordance with our earlier work (Royse *et al.*, 1983a).

In our earlier work (Royse *et al.*, 1983a), we did not observe isolates of *L. edodes* polymorphic for *Mdh-2*. However, in this study one additonal allele (115) at *Mdh-2* (Fig. 1) was observed in an isolate (line 604, genotypic class 38) from Korea. This locus, therefore, was added to the list of loci used to catalog isolates. The locus coding for alkaline phosphatase (AKP), reported in our earlier work (Royse *et al.*, 1983a), was not used in this study because it was the most difficult enzyme of the group to resolve.

The inferred genotypic bases of the electrophoretic phenotypes for the isolates examined in this study are presented in Table III. Thirty-eight genotypic classes were recognized. Genotypic class 2 contains several isolates of *L. edodes* that, in most cases, could be traced to a common historical source. Genotypic class 6 contains isolates that are commonly used in Taiwan, Japan, and Korea for shiitake production on sawdust. Genotypic classes 5, 32, and 33 contain hybrids Le85, Le53, and Le65, respectively.

A UPGMA cluster analysis (Sneath and Sokal, 1973), based on Rogers' (1972) coefficients of similarity was performed and is presented in Fig. 2. These are relative similarities because we chose only multiallelic loci. Genotypic classes 35–38 may represent another species because they have multiple alleles not found in other isolates. All these lines clustered below 0.6 similarity.

DISCUSSION

The ability to catalog lines of *L. edodes* is paramount to the breeding effort required to improve the productivity of this species. Due to the long-standing tradition in the mushroom industry to renumber isolates, rather than to use a consistent system of naming or numbering, breeding efforts have been impeded. In the absence of methodology to catalog lines, duplicate cultures with different numbers are often evaluated independently for potential use in deriving breeding stock. Therefore, considerably more effort and resources are required to make the same genetic improvements through breeding and

Table III. Inferred Genotypes of 91 Isolates of *Lentinula edodes* in The Pennsylvania State University Mushroom Culture Collection (PSUMCC)

Genotypic class no.	Locus[a]											Line no. in PSUMCC
	Gpi	*Pgk*	*Pep-1-* LLL	*Aat-1*	*Sod*	*Mpi*	*Dia*	*Mdh-1*	*Mdh-2*	*Gdh*	*Pep-2-* GL	
1	11	22	11	12	11	11	11	11	11	11	11	295, 473, 652
2	11	22	12	12	11	11	11	11	11	11	11	30, 72, 298, 321, 369, 382, 383, 392, 394, 451, 452, 453, 474, 487, 563, 564, 641, 643
3	11	22	12	12	11	11	11	11	11	13	11	651
4	11	22	11	22	11	11	11	11	11	13	11	378
5	11	22	12	22	11	11	11	11	11	13	11	324, 325, 379, 645, 647
6	11	22	12	22	11	11	11	11	11	11	11	400, 468, 469, 565, 588, 592, 593
7	11	22	11	13	11	11	11	11	11	13	11	642
8	11	22	11	22	22	11	11	11	11	11	11	61
9	11	22	11	22	12	11	11	11	11	11	11	60, 268
10	12	22	11	12	12	11	11	11	11	11	11	389, 390
11	11	22	11	22	11	11	12	14	11	11	11	464
12	12	12	11	12	11	11	11	11	11	13	11	131
13	12	12	11	12	11	11	11	11	11	14	11	385, 386
14	12	12	11	11	11	11	11	11	11	11	11	470, 648
15	12	13	11	12	11	11	11	11	11	11	11	380, 471

16	11	22	11	11	22	11	11	11	11	11	12	322
17	11	23	11	11	22	11	11	11	11	11	22	296
18	11	22	11	11	11	11	11	11	11	11	22	297, 488, 653
19	11	22	11	12	11	11	11	12	11	11	12	Le85[b]
20	11	23	11	12	11	11	11	12	11	11	12	293, 318, 666
21	11	23	11	12	11	11	11	12	11	11	11	323, 375, 387, 388, 463, 542
22	11	23	11	12	11	11	11	11	11	11	11	543, 649
23	11	23	11	12	11	11	11	11	11	11	22	545, 590
24	11	23	11	12	11	11	11	11	11	11	12	305
25	12	23	11	12	12	11	11	11	11	11	12	304, 376
26	11	23	11	12	12	11	11	12	11	11	12	320, 393
27	11	23	11	11	12	11	11	11	11	11	12	377, 472, 489, 490, 544, 589, 603, 650
28	11	33	11	11	11	11	11	11	11	11	11	591
29	11	33	11	22	11	11	11	11	11	11	11	381, 391
30	11	11	11	11	22	11	11	11	11	11	11	294
31	22	11	11	11	13	11	11	11	11	11	11	384
32	12	12	11	11	11	11	11	22	11	11	22	Le53[b]
33	12	13	11	12	11	11	11	22	11	11	22	Le65[b]
34	11	33	11	22	11	22	12	11	11	14	22	291, 292
35	11	44	66	11	11	66	11	33	11	44	11	585
36	11	44	66	11	11	66	11	23	11	14	11	587
37	11	44	66	11	11	66	11	33	11	11	11	675
38	11	44	66	11	11	66	11	33	22	44	11	604

[a] See Fig. 1 for phenotypes.
[b] Hybrids.

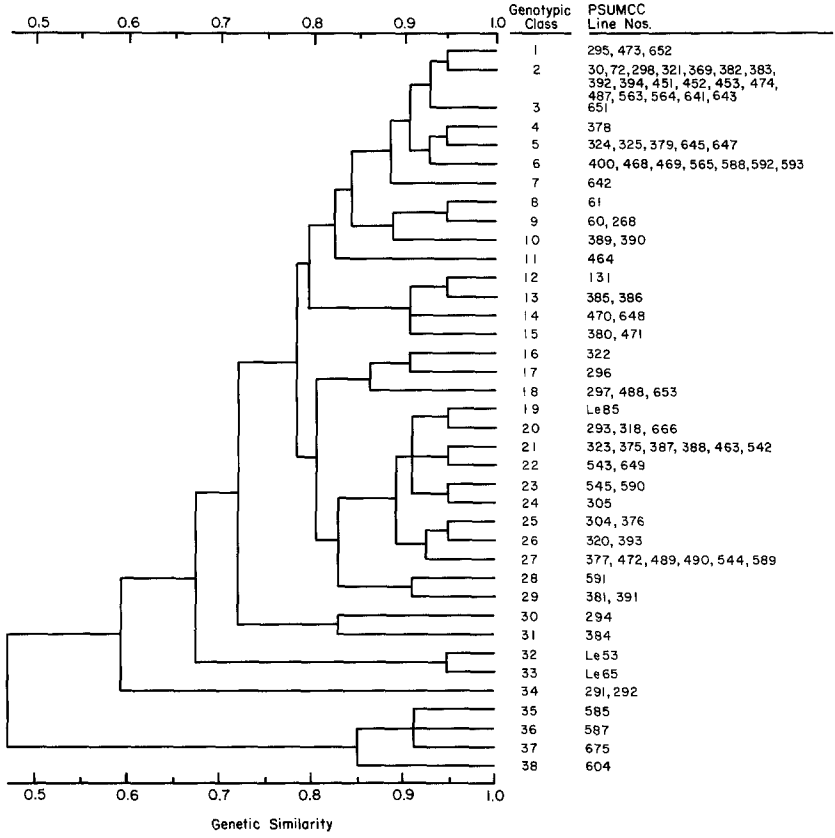

Fig. 2. A UPGMA cluster analysis (Sneath and Sokal, 1973) of *Lentinula edodes* genotypes based on Rogers' (1972) coefficients of similarity.

selection. As can be seen from the results of this study, approximately 60% of the isolates obtained from worldwide sources are probably duplicates.

In the absence of a system to identify and protect newly developed lines, incentives to develop new cultivars are practically nonexistent. The tradition in the spawn industry is to borrow competitors' lines freely, by way of tissue culture, and then to propagate the genetic material, providing it with a new number (San Antonio, 1984; Eger, 1979; Bels, 1962). A case in point is the worldwide use of *Agaricus bisporus* (Lang) Imbach hybrids developed at Horst (U_1 and U_3) by Fritsche (1983). These hybrids are now available from nearly 100% of the spawnmakers, although only one major spawn-producing company currently is providing royalities to Horst. Japan is facing similar problems with shiitake germplasm borrowing and has recently instituted a germplasm registration system (Royse *et al.*, 1985; Anonymous, 1980a, b).

While patents of mushroom cultivars have been granted in the past (San Antonio, 1984; Lambert, 1962; Robbins, 1961), they have generally been considered to have little importance because of the difficulty in differentiating the patented lines from existing cultivars. This difficulty has been overcome with the system of identification reported by Royse and May (1986a, b). Our system should provide a practical, unambiguous system of identifying shiitake lines. An unambiguous identification system will become more important in the future, as increasing interest is shown in patenting microorganisms (Cooper, 1982; Crespi, 1982; Bagwill, 1981; Anonymous, 1979). The crosses (Le53, Le65, and Le85) developed as part of this study show the ability to produce unique, identifiable germplasm. Following the completion of performance evaluations, these newly generated lines, if superior, can be patented, licensed, and released for commercial use.

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