PLANT GENETIC RESOURCES

The Genetic Anatomy of a Patented Yellow Bean

L. Pallottini, E. Garcia, J. Kami, G. Barcaccia, and P. Gepts*

ABSTRACT

Since a 1980 Supreme Court decision, it is possible in the USA to obtain a utility patent for crop cultivars and other life forms. Furthermore, it is also possible to obtain Plant Variety Protection (PVP) for a cultivar. Among the awards of the United States Patent and Trademark Office and the USDA Plant PVP Office are a utility patent and a PVP certificate, respectively, associated with a yellow-seeded bean (*Phaseolus vulgaris* L.), specifically the cultivar Enola. These awards have been controversial because of, among several reasons, the perceived lack of novelty of the yellow seed color and the cultivar itself. To check the origin of Enola, we fingerprinted a representative sample of 56 domesticated common bean accessions, including a subsample of 24 cultivars with yellow seeds similar to those of Enola. Fingerprinting was accomplished with amplified fragment length polymorphisms (AFLP). Five *Eco*RI/*Mse*I and five *Pst*I/*Mse*I primer combinations were used, which revealed 133 fragments. The *Pst*I/*Mse*I primer combinations revealed a 3-fold larger number of polymorphic markers than the *Eco*RI/*Mse*I primer combinations. Most yellow-seeded beans, including Enola, were included in a tightly knit subgroup of the Andean gene pool. Enola was most closely related to the pre-existing Mexican cultivar Azufrado Peruano 87. A sample of 16 individuals of Enola displayed a single 133-AFLP-fragment fingerprint, which was identical to a fingerprint observed among yellow-seeded beans from Mexico, including Azufrado Peruano 87. Probability calculations of matching the specific Enola fingerprint showed that the most likely origin of Enola is by direct selection within pre-existing yellow-bean cultivars from Mexico, most probably 'Azufrado Peruano 87'.

In 1980, the U.S. Supreme Court instated (447 U.S. 303) the award of a utility patent for a genetically engineered *Pseudomonas* bacterium capable of breaking down crude oil (U.S. Supreme Court, 1980). An application for this patent had initially been rejected by the U.S. Patent and Trademark Office (PTO) on the ground that living things are not patentable subject matter according to the statute governing patents (Title 35 U.S.C. 101). The landmark Supreme Court decision initiated a new era in which patents for life forms, including DNA sequences, cell lines, transgenic animals and plants, and crop cultivars, could be obtained in the USA. Before this decision, the only life form for which a patent could be obtained were vegetatively propagated plants (so called plant patents). Utility patents are awarded for inventions that are novel, in that they must not have been made public for more than 1 yr. They should also be useful and nonobvious to someone skilled in the art (35 USC § 101, 102, 103) (U.S. House of Representatives, 2002).

In 1999, the U.S. PTO awarded patent no. 5,894,079 for the yellow-seeded cultivar Enola of common bean. The main claim of this patent is the yellow color of the seed coat of Enola. According to the patent description (Proctor, 1999), seeds of this cultivar had been obtained as part of a mixed bag of seeds of different colors purchased in Mexico in 1994. The yellow seeds were then planted in a field in Colorado for 3 yr (1994–1996) after which a patent for this yellow-seeded variety was filed on 15 Nov. 1996. Furthermore, the Plant Variety Protection (PVP) Office of the USDA issued PVP certificate no. 9700027 for cultivar Enola in 1999 (http://www.ars-grin.gov/cgi-bin/npgs/html/acchtml.pl?1536394; verified 8 January 2004). The award of these intellectual property rights has generated widespread attention in the media (New York Times: Pratt, 2001; National Public Radio: Tolan, 2001; Wall Street Journal: Friedland, 2000).

Yellow beans are among traditional bean cultivars grown principally in Mexico and Peru under several names such as Azufrado and Canario (Voysest, 2000). Originally, cultivars from these two countries represented two evolutionarily distinct groups of cultivars as they originated from two different domestications, one in Mexico and the other in the southern Andes (Gepts et al., 1986). More recently, Mexican bean breeders developed a new commercial class of yellow-seeded bean cultivars called Azufrado Peruano or Peruano by crossing yellow-seeded bean cultivars from Mexico with those of Peruvian origin (Voysest, 2000). Beans with yellow-colored seeds such as the Peruano types are grown and consumed mainly in the northwestern part of Mexico (Anonymous, 2000), but Mexican immigration has created a market for yellow-seeded beans in the USA.

We investigated here whether the Enola bean represents a distinct cultivar compared with the existing Peruano or other yellow-seeded cultivars from Mexico. We conducted DNA fingerprinting experiments to determine the relationships between Enola and other bean cultivars, including a sample of other yellow-seeded cultivars. We calculated probabilities of obtaining a match for the Enola fingerprint under different breeding scenarios. Furthermore, we compared the leaf color of Enola with that of selected yellow-seeded cultivars because

L. Pallottini and G. Barcaccia, Department of Agronomy and Crop Science, University of Padova, Agripolis, Via Romea 16, 35020 Legnaro, PD, Italy; E. Garcia, Department of Food Science and Technology, University of California, 1 Shields Avenue, Davis, CA 95616-8598, USA; J. Kami and P. Gepts, Department of Agronomy and Range Science, University of California, 1 Shields Avenue, Davis, CA 95616-8515, USA. Received 9 July 2003. *Corresponding author (plgepts@ucdavis.edu).

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677 S. Segoe Rd., Madison, WI 53711 USA

Abbreviations: AFLP, amplified fragment length polymorphism; AP78, Azufrado Pimono 78; AP87, Azufrado Peruano 87; ATCC: American Type Culture Collection; INIFAP: Instituto Nacional de Investigaciones Forestales y Agropecuarias, Mexico; PRO: Proprietary source; PTO, Patent and Trademark Office; PVP, Plant Variety Protection; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; UC Davis: University of California, Davis.

the PVP application for Enola cited leaf color difference as a distinguishing mark between Enola and Azufrado Pimono 78, the original yellow-seeded cultivar of the Peruano type in Mexico. Our results show that the DNA fingerprint of Enola is identical to a fingerprint found in Mexican yellow-seeded beans of the Peruano group.

MATERIALS AND METHODS

Plant Materials

In a first experiment, a sample of 56 entries was established to investigate the relationships of cultivar Enola with other common bean cultivars (Table 1). One individual of each entry was analyzed. Some of the entries in this sample were received from more than location or more than once. Because they were analyzed separately, they were counted as a distinct entry. Enola seeds were obtained from the American Type Culture Collection, the official repository for patented cultivars, as well as from a private source. The six major races of common bean (Singh et al., 1991a) were represented by five to six accessions each, chosen on the basis of previous molecular markers analyses (Gepts, 1984, 1988; Singh et al., 1991b). In addition, special attention was devoted to assembling a sample of cultivars, whose seeds show a yellow color similar to that of Enola (Fig. 1). These included the following materials: (i)

Table 1. Common bean materials analyzed in this study.

Entry No.	CIAT No.	Name	Alternate designations	Country†	State‡	Source§
1¶		Enola 2000		USA		ATCC
2¶		Canario 707		USA		Steve Temple, UC Davis
3	G02400	Mantequilla	Gentry 21953; PI312090	MEX	SON	CIAT
4	G03273	Morado de Aguascalientes	AGS-74-B	MEX	AGS	CIAT
5	G03290	Flor de Mayo	AGS-88	MEX	AGS	CIAT
6	G03504	Ojo de Cabra	CHIH-31; X-15267	MEX	CHI	CIAT
7	G03715	Porrillo-1		ELS		CIAT
8	G04390	Pinto	TLAX-51	MEX	TLX	CIAT
9	G04456	Jamapa		MEX	VER	CIAT
10	G04471	Cristal Blanco		CLE		CIAT
11	G04474	Coscorrón		CLE		CIAT
12	G04666	Magdalena 3		COL	MAG	CIAT
13	G04922	Rojo de Seda		HDR		CIAT
14	G05024	Jalo	BZL-0237; collected 1935	BRA		CIAT
15	G05036	Mulatinho		BRA		CIAT
16	G05254	Bagajo		BRA		CIAT
17	G05910	Burros Grandes		CLE		CIAT
18	G06861	Bayo		HDR		CIAT
19	G07385	Uribe Redondo		COL		CIAT
20	G08159	Radical		COL		CIAT
21	G11013	Bayo		MEX	DUR	CIAT
22	G11295	Frijola	GTO-55-2; MEX-187	MEX	GTO	CIAT
23	G11511	Frutilla	CLE-027	CLE		CIAT
24	G11733	Caballero		PER		CIAT
25¶	G11891	Culiacán	CULIACAN-11-57R-M-37-M-M	MEX	SIN	CIAT
26	G12717	Bolón Rojo		COL	NAR	CIAT
27	G19068	Apetito	JAL-4; PI313367	MEX	JAL	CIAT
28¶	G13094	Mayocoba	collected 1959	MEX		CIAT
29	G20553	Conejo	NVRS-431	MEX		CIAT
30	G19646	Quqa Pava		PER	CAJ	CIAT
31	G21720	Cargabello		COL		CIAT
32	G22041	Garbancillo Zarco		MEX		CIAT
33¶	G22215	II8FR MO-5-3-M-2-1-M		MEX		CIAT
34¶	G22227	MO-85-86 2598	SIN 9	MEX	SIN	CIAT
35¶	G22230	MO-85-86 2780	SIN 12	MEX	SIN	CIAT
36	G24554	Tórtolas Corriente		CLE		CIAT
37	G50517	G50517	OT-646; Cargamanto	COL	ANT	CIAT
38¶		Woodland Yellow		USA	NEB	J. Kami
39		BAT93				CIAT
40		Jalo EEP558		BRA		CIAT
41¶		Sulphur BN142	= A	USA		J. Nienhuis and K. Kmiecik, University of Wisconsin
42¶		Mayocoba 1998		USA		PRO
43¶		Mayocoba 2001		USA		PRO
44¶		Myasi 2001		USA		PRO
45¶		Frijol Canario		PER		P. Gepts
46¶		Azufrado Peruano 87		MEX		J. Acosta (INIFAP)
47¶		Azufrado Regional 87		MEX		J. Acosta (INIFAP)
48¶		Azufrado Regional 87		MEX		INIFAP
49¶		Azufrado Peruano 87		MEX		INIFAP
50¶		Azufrado Pimono78		MEX		INIFAP
51¶		Enola 2001		USA		PRO
52¶		Enola 2000-2		USA		ATCC
53¶		Enola-PRO	= B	USA		PRO
54¶		Mayocoba	= C	USA		PRO
55¶		Myasi	= D	USA		PRO
56¶		Enola 2002	= E	USA		ATCC

† ISO country codes: BRA: Brazil; CLE: Chile; COL: Colombia; ELS: El Salvador; HDR: Honduras; MEX: Mexico; PER: Peru; USA: United States of America.

‡ State, province, or department code: AGS: Aguascalientes; ANT: Antioquia; CAJ: Cajamarca; CHI: Chihuahua; DUR: Durango; GTO: Guanajuato; JAL: Jalisco; NAR: Nayarit; NEB: Nebraska; SON: Sonora; TLX: Tlaxcala; VER: Veracruz.

§ Sources: ATCC: American Type Culture Collection; INIFAP: Instituto Nacional de Investigaciones Forestales y Agropecuarias, Mexico; PRO: Proprietary source; UC Davis: University of California, Davis.

¶ Indicates a yellow-seeded cultivar.

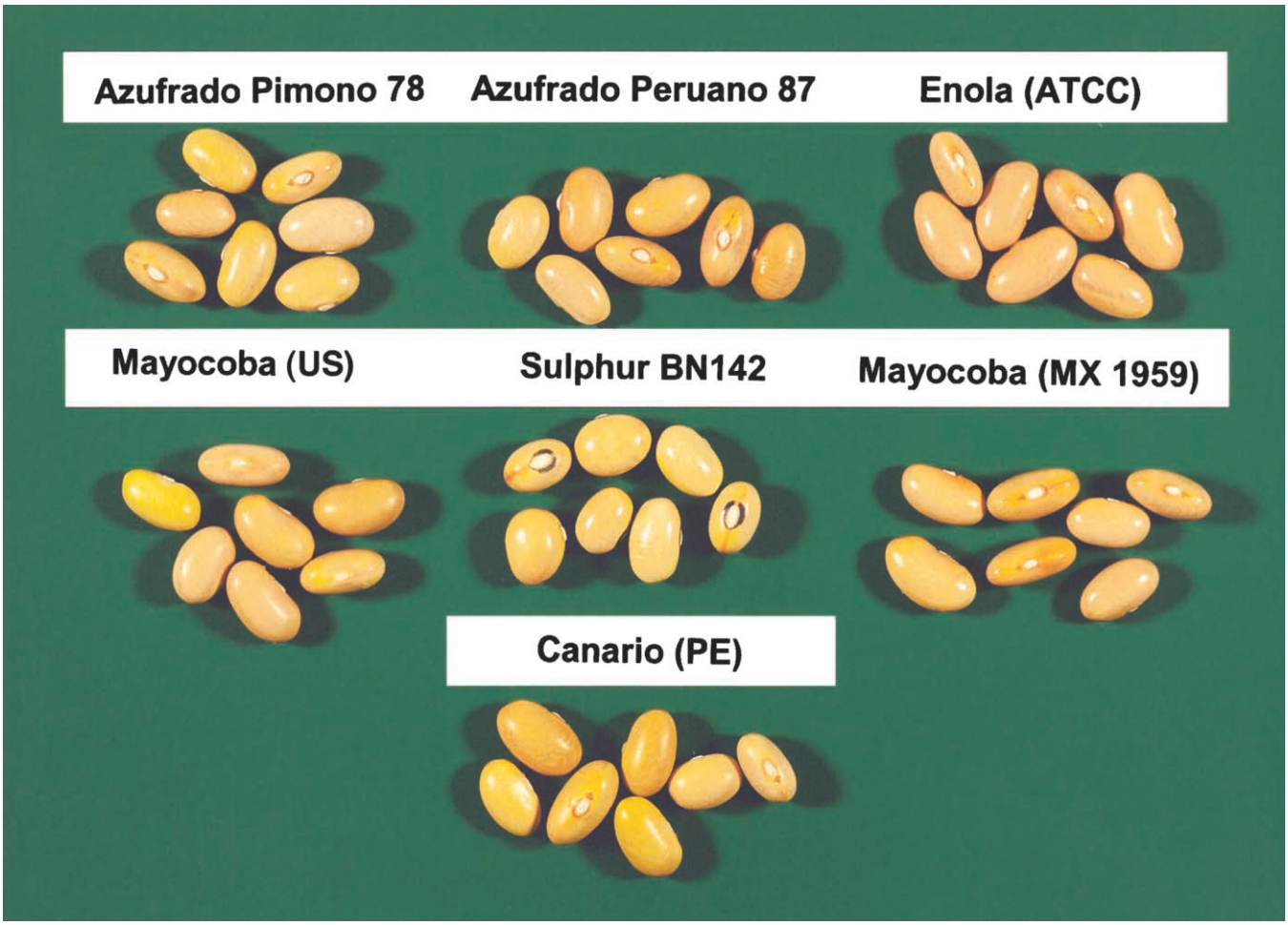

Fig. 1. Yellow-seed cultivars analyzed in this study. Mayocoba (MX 1959) and Canario (PE) are representative of the parents that gave rise to the Peruano type cultivar class. Examples of this class are the Mexican cultivars Azufrado Pimono 78 (the original Peruano-type cultivar released in 1978) and Azufrado Peruano 87 (released in 1987) (Voysest, 2000). Enola is a patented and PVP certified yellow-seeded cultivar (Proctor, 1999; http://www.ars-grin.gov/cgi-bin/npgs/html/acchtml.pl? 1536394; verified 27 January 2004). Sulphur BN142 is an ancient U.S. cultivar described as early as 1931 (Hedrick 1931).

Mexican cultivars belonging to the Peruano type commercial class, including Azufrado Pimono 78, the original cultivar in this class (Voysest, 2000), and Azufrado Peruano 87, a cultivar released in 1987 (Fig. 1); (ii) breeding lines in the Peruano category, such as SIN9 and SIN12; (iii) putative representatives of the Andean and Mesoamerican seed classes that gave rise to the Peruano class, Canario and G13094 (Mayocoba), respectively (Fig. 1); and (iv) the heirloom cultivar Sulphur BN142, described by Hedrick (1931) (Fig. 1).

In a second experiment, 15 individuals of each of three entries were analyzed. These entries included Enola (obtained from ATCC), Mayocoba (from a proprietary source), and Azufrado Peruano 87 (from INIFAP, Mexico). Results of the 15 individuals from this experiment were then combined with those of the respective individual analyzed in the first experiment, giving a total of 16 individuals for each entry. To allow a blind test in both experiments, individual accessions were given a consecutive number (Table 1) after receipt in the laboratory. This number was used throughout the experimentation and analysis of the results to allow a blind analysis of the results.

Amplified Fragment Length Polymorphisms

DNA was extracted from leaves harvested before flowering from greenhouse-grown leaves as described by Gepts and Clegg (1989), but without the addition of polyvinylpolypyrrolidone. Amplified fragment length polymorphisms were analyzed as described by Vos et al. (1995) and modified by Barcaccia et al. (1999). The primer combinations included five *Eco*RI-*Mse*I (with selective bases CAC/AAG, CAC/AGC, CCA/AGA, CCA/AGC, and CAA/AAG) and five *Pst*I-*Mse*I combinations (AG/CAC, AG/CAT, AG/CCA, AT/CAA, and AT/CAC).

To compare the efficiency of *Eco*I/*Mse*I and *Pst*I/*Mse*I primer combinations, an assay efficiency index (AI) was calculated (Table 2). The index relies on the effective number of alleles identified per locus, determined as $n_e = 1/\Sigma(p_i^2 + q_i^2)$, where p_i and q_i are the frequencies of marker alleles, present vs. absent, respectively at the ith locus. The index is then computed as $AI = (\Sigma n_e)/P$, where Σn_e is the total number of effective marker alleles detected over all polymorphic loci and P is the total number of assays performed (i.e., the number of primer combinations used) for their detection (Porceddu et al., 2002).

Data Analysis

Multivariate Analyses

In the first experiment, the principal coordinate analysis was implemented by, in succession, the SIMQUAL, DCENTER,

Table 2. Levels of polymorphism identified by *Eco*RI/*Mse*I and *Pst*I/*Mse*I primer combinations of AFLP markers.

Primer combinations	All AFLP fragments			Polymorphic AFLP fragments			Assay efficiency index
	Total number	Mean no. per primer pair	Proportion of total number (%)	Total number	Frequency of polymorphism (%)	Proportion of total polymorphic number (%)	
*Eco*RI/*Mse*I†	314	63	46	34	11	26	11
*Pst*I/*Mse*I†	376	75	54	99	26	74	31
Totals	690	69		133	19		21

† 5 primer combinations (see Materials and Methods).

EIGEN, and 3DPLOT programs of NTSYS (Rohlf, 1997). For the second experiment, genetic similarities expressed as Dice's coefficient were calculated using the SIMQUAL program of NTSYS. A dendrogram was then calculated on the basis of the Unweighted Paired Group Method using Arithmetic averages algorithm implemented in the SAHN program of NTSYS. Bootstrap values of the clusters were calculated on the basis of 10 000 replications with the WINBOOT program (http://www.irri.org/textonly/science/software%20downloads/winboot.htm; verified 27 January 2004).

Calculation of DNA Fingerprinting Profile Probabilities

Five possible breeding scenarios were considered to have given rise to cultivar Enola. Three of these involved hybridization between inbred (homozygous) cultivars and two involved selection within a Peruano-type cultivar (see Results section). For each of the three scenarios involving cultivar hybridizations (Table 3), the probabilities of obtaining the Enola profile were calculated as $\tilde{P} = \prod_i \tilde{p}_i^2$ (Weir 1996, p. 218), with $\tilde{p}_i$ being the probability of obtaining the ith fragment state observed for the Enola profile (either presence or absence of the fragment). This formula is valid only if AFLP fragments show independence among each other. We defined independent markers as those markers for which less than 10% of the Fisher exact tests for independence with all other markers were statistically significant ($P \leq 0.10$). For the first scenario (a cross between any Andean and Middle American cultivars):

$$\tilde{p}_i = \begin{cases} u_i^A u_i^M + 0.5 u_i^A v_i^M + 0.5 v_i^A u_i^M \\ v_i^A v_i^M + 0.5 v_i^A u_i^M + 0.5 u_i^A v_i^M \end{cases}$$

for the presence or absence of fragment i, respectively, where u_i^A, v_i^A, u_i^M, and v_i^M 0 are the frequencies of the presence (u) or absence (v) of fragment i in the Andean (A) and Mesoamerican (M) gene pools, respectively. For the second scenario [a cross between representatives of yellow-seeded cultivars of the Andean (Frijol Canario) and Middle American (Mayocoba: G13094) gene pools]:

$$p_i = \begin{cases} 1 \\ 0.5 \end{cases}$$

when both parents show the same or different fragment i state (present or absent), respectively, as the Enola profile. For the third scenario (cross between members of the group of yellow-seeded cultivars):

$$\tilde{p}_i = \begin{cases} (u_i^Y)^2 + u_i^Y v_i^Y \\ (v_i^Y)^2 + u_i^Y v_i^Y \end{cases}$$

for the presence or absence of fragment i, respectively, where u_i^Y and v_i^Y are the frequencies for the presence (u) and absence (v) of the ith fragment among yellow-seeded beans. For the fourth and fifth scenarios involving selections within existing Peruano-type cultivars, the probability of the Enola profile was calculated as $\tilde{P} = n_A/n$ 0, where n_A is the frequency of individuals with the Enola marker profile in the sample of size n of the respective cultivars. Variances for the crossing scenarios were calculated as in Weir (1996, p. 218) and those for the selection scenario as $n_A (1 - n_A)/n$. For all scenarios, homozygosity of the cultivars was assumed on the basis of the predominantly self-pollinating nature of *P. vulgaris*.

Leaf Color Analysis

Leaf color was analyzed in a greenhouse experiment, planted on 14 May 2002. Yellow-seeded entries (No. 42–52 of Table 1) were included in the experiment. Color observations were made on 4 and 5 June 2002. At those dates, plants had a fully expanded first trifoliolate and an expanding second trifoliolate. The experimental design was a randomized complete block design with three replicates. The experimental unit was a single pot with four plants. Three measurements were made on the first trifoliolate of each plant. The three measurements were then averaged and further statistical calculations were based on these averages. Leaf color measurements were conducted with a Minolta Chroma Meter CR-200 (Minolta, Ramsey, NJ), a tristimulus colorimeter, calibrated with a standard white tile (Y 94.6 x 0.3143 y 0.3209) and a standard green tile (Y 34.0 x 0.2770 y 0.3650). Results were reported in L*, chroma ($\sqrt{a^{*2} + b^{*2}}$) and hue angle (arctangent b*/a*). The value L* is a measure of lightness, it ranges from 0 (black) to 100 (white). The value L* is a measure of lightness, it ranges from 0 (black) to 100 (white). Chroma is a measure of color saturation or intensity, and hue angle denotes the color (an angle of 0° corresponds to red-purple, 90° to yellow, 180° to bluish-green and 270° to blue (McGuire, 1992).

Results for the three variables—Lightness (L), Chroma (C), and Hue Angle (h)—were first examined for a fit to a normal distribution using the PROC UNIVARIATE procedure of SAS. All three variables exhibited normality according to a Shapiro-Wilk test ($r > 0.95$). Differences among cultivars

Table 3. Probability of the AFLP marker profile shown by cultivar Enola assuming various hypothetical breeding scenarios.

Breeding scenario†	Number of independent markers	Probability	Variance
Cross between any Andean and Mesoamerican cultivar analyzed in this study	31	1×10^{-18}	2×10^{-18}
Cross between original yellow-seeded Middle American (Mayocoba, G13094) and Andean (Frijol Canario) cultivars	31	3×10^{-14}	5×10^{-12}
Cross between any pair of yellow-seeded cultivars	24	3×10^{-5}	2×10^{-4}
Selection without crossing from:			
Mayocoba	–	6×10^{-2}	4×10^{-3}
Azufrado Peruano 87	–	3×10^{-1}	1×10^{-3}

† See text for further explanations on the scenarios.

were analyzed by the PROC GLM procedure of SAS using a mixed model, with cultivars as a fixed factor and replicates as the random factor, and a Type III expected mean square. Following rank transformations, comparisons among means were conducted based on least squares means, adjusted for multiple comparisons by the Tukey-Kramer procedure.

RESULTS

Levels of Polymorphism Observed with AFLPs

In a first experiment, we used AFLP markers (Vos et al., 1995) as modified (Barcaccia et al., 1999) to determine the genetic relationships among bean cultivars. A panel of 56 bean entries was analyzed (Table 1) and consisted of 32 cultivars representative of the genetic diversity of beans in general (Singh et al., 1991a) and 24 cultivars with yellow seeds similar to those of the cultivar Enola (see Materials and Methods).

The 10 AFLP primer combinations (five *Eco*RI/*Mse*I and five *Pst*I/*Mse*I) revealed 133 polymorphic amplified fragments among 690 amplified fragments (19% polymorphism) in the sample of Andean and Mesoamerican accessions (Table 2). There was a marked difference between the *Eco*RI/*Mse*I primers and *Pst*I/*Mse*I primers in the number of polymorphic markers identified. *Pst*I/*Mse*I primers produced a slightly larger proportion of fragments compared to *Eco*RI/*Pst*I primers (54 vs. 46%). However, the frequency of polymorphic fragments was substantially higher among the former compared to the latter (26% vs. 11%). Taking into account both the total number of amplified fragments and the level of polymorphism, *Pst*I/*Mse*I primers were three-fold more powerful in detecting polymorphisms than the *Eco*RI/*Mse*I primers (76 vs. 24%, respectively, of the total number of polymorphic bands detected in this experiment; Assay Efficiency Index (Porceddu et al., 2002) of 31 vs. 11, respectively) (Table 2).

Genetic Relationships in a Representative Sample of Common Bean Cultivars

The first three coordinates of the principal coordinates analysis of AFLP markers explained 58, 7, and 5%, respectively, of the variation observed (Fig. 2). The first principal coordinate separated Middle American (negative coordinates) from Andean (positive) bean domesticates as previously observed (Gepts et al., 1986; Singh et al., 1991a). Mean genetic similarity estimates within the two groups were 0.90 (Andean gene pool) and 0.77 (Middle American gene pool), whereas between them it was 0.43, confirming the existence of a major genetic differentiation between the two gene pools. The second principal coordinate separated the Mesoamerica race (positive coordinates) from races Durango and Jalisco (negative coordinates, Fig. 2) (Singh et al., 1991a). However, a high level of genetic similarity was observed between these two subgroups (0.74). The third axis separated the yellow-seeded group (positive coordinates) of the Peruano type from the rest of the Andean cultivars (negative coordinates). Within the Mesoamerican group, a yellow-seeded cultivar (G13094) is a representative of the Mesoamerican parent of the Peruano class. It was collected in 1959 before breeding programs were initiated that led to the development of the Peruano cultivars in Mexico. A representative of the Andean parent of the Peruano class is Frijol Canario, situated as expected within the Andean group in Fig. 2.

Within the yellow-seeded Andean group, the cultivar Enola (obtained from its official source at the American Type Culture Collection) was part of a tightly knit group including the Mexican cultivar Azufrado Peruano 87 as well as the U.S. cultivar Myasi (represented by two samples, Myasi and Myasi 2001), suggesting a very close genetic relationship, if not an identity, between these cultivars (Fig. 2). Of particular importance is the comparison between Enola and Azufrado Peruano 87 given the Mexican origin of Enola as stated in the patent (Proctor, 1999) and the PVP certificate (U.S. Department of Agriculture, http://www.ars-grin.gov/cgi-bin/npgs/html/acchtml.pl?1536394; verified 8 January 2004). The individual of Enola that was tested was identical or differed by a single fragment from the 133-fragment AFLP profile of the individual from Azufrado Peruano 87 depending on the source of the latter cultivar. Additional slight differences in fingerprinting pattern among different samples of the same variety (e.g., Azufrado Regional, Mayocoba) suggested that low levels of AFLP polymorphism could be present in cultivars of common bean.

Variability within Yellow Bean Cultivars

Therefore, a second experiment was conducted to confirm the fingerprinting patterns established in the first experiment, to assess the level of intracultivar AFLP polymorphism, and further investigate the relationship between Enola and Azufrado Peruano 87. AFLP fingerprinting was conducted on a sample of 16 individuals each for three cultivars (including one individual analyzed already in the previous experiment as a positive control). In addition to Enola and Azufrado Peruano 87 (entries 1 and 49 in Table 1), a sample of the U.S. proprietary cultivar Mayocoba harvested in 1998 was also included. The number of individuals analyzed per cultivar (16) was chosen in part to run all three samples side by side on the same acrylamide gel to facilitate comparisons.

Thirty-two of the same 133 fragments observed in the first experiment were polymorphic. The Enola sample was monomorphic; all 16 individuals of Enola showing the same fragment profile (Fig. 3). The Mayocoba 1998 sample showed five different combinations of fragments among 16 individuals, with one individual each harboring the same profile as in the Enola and Azufrado Peruano 87 samples, respectively. The Azufrado Peruano 87 sample exhibited eight combinations. Five of the individuals showed the same profile as in Enola.

A cluster analysis (Rohlf, 1997) of the 48 individuals analyzed confirmed that Azufrado Peruano 87 was more closely related to Enola than Mayocoba 1998 (Fig. 3). Fourteen of the sixteen individuals of Mayocoba clustered in a group that was highly supported by a bootstrap analysis (score of 96). The sixteenth individual tested of this cultivar had the same combination of AFLP

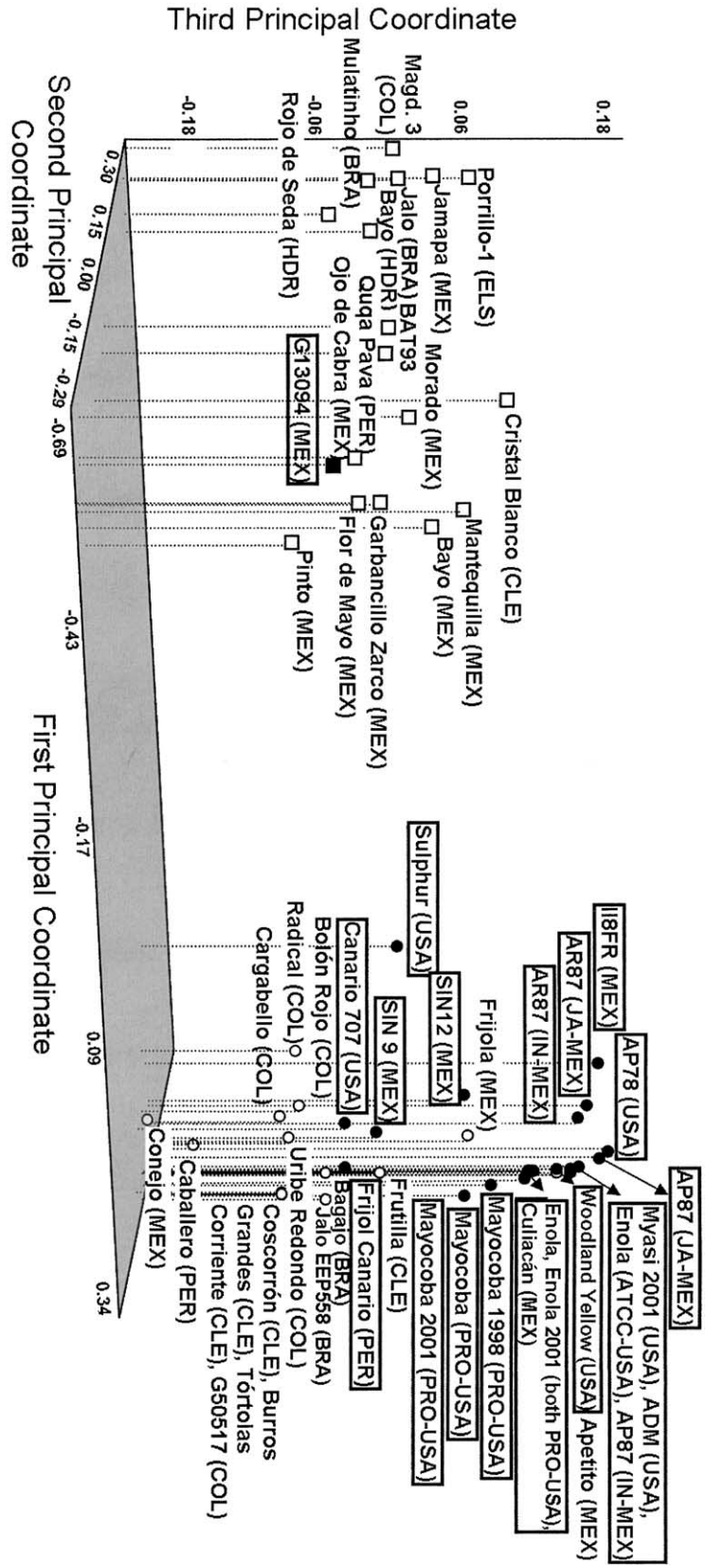

Fig. 2. Principal coordinate analysis of AFLP diversity in a sample of 56 common bean cultivars. Square symbols: Middle American gene pool; circles: Andean gene pool. Boxed entries and filled symbols: yellow seed coat entries. AP78: Azufrado Peruano 78; AP87: Azufrado Peruano 87; AR: Azufrado Regional 87. The eigenvalues of the three axes are 58, 7, and 5%. For further explanations about the identity of the different groups, see text. For the identity of each entry, see Table 1.

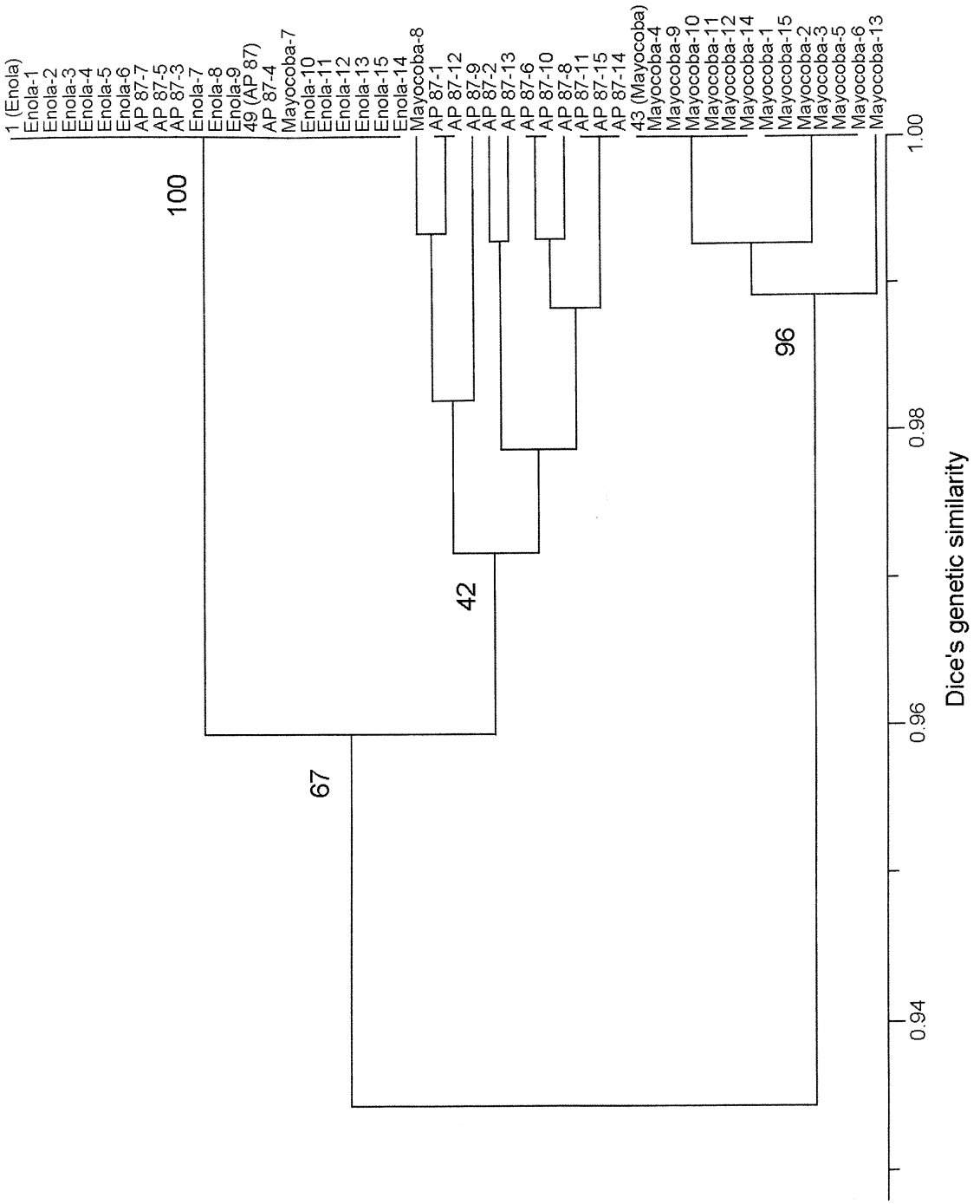

Fig. 3. UPGMA dendrogram showing the relationships among AFLP fragment profiles found in three Peruano-type bean cultivars: Enola, Azufrado Peruano 87 (AP87), and Mayocoba 1998. Each branch represents a different combination. Individuals to the right of vertical bars have identical combinations. The numbers within the tree are bootstrap values.

fragments as Enola. Cultivars Enola and Azufrado Peruano 87 formed a joint cluster with moderate to high support (score of 67). However, the poor support (score of 42) for an Azufrado Peruano 87 cluster separate from an Enola cluster suggests that the two samples are not significantly distinct. This can be attributed to the overall similarity among the different genotypes characterizing this group (Dice genetic similarity >0.96, Fig. 3), as well as the fact that the Enola profile was identical to that of five individuals of the sample of Azufrado Peruano 87.

Probabilities of Obtaining the Enola Fingerprint under Different Breeding Scenarios

Several scenarios were considered to account for the possible origin of Enola (Table 3). Three of these involved hybridization between genotypes of different evolutionary origins and two involved selection and

pure-line development within existing yellow-seeded (sub)populations. The hybridization scenarios reflect information about the origin of the Peruano commercial type, whereas the selection within yellow bean population scenarios reflected the information contained in the Enola patent and PVP certificate. Both major geographic gene pools of common bean contain yellow beans, generally called Canarios in Peru and Azufrados in Mexico. Hybridization between these two classes by Mexican bean breeder H. López, with the collaboration of F. Hernández, lead to the creation, with the release in 1978 of Azufrado Pimono 78, of a new commercial class, the Azufrados Peruanos (Voysest, 2000). This new class has a more compact growth habit and more intensely yellow seed. It is also possible, however, that yellow beans can appear by recombination between parents, one or both of which do not have yellow seeds (S. Temple, pers. comm.). The seed color genotype responsible for the yellow seed color characteristic of the Peruano types consists of seven genes. Thus, a cross between complementary genotypes at these seed color genes can also lead to yellow-seeded progeny. A third hybridization scenario reflects hybridization between two preexisting, yellow-seeded beans of the Peruano type. Both the patent and the PVP certificate describe how the Enola cultivar was developed by selection within an existing yellow bean population acquired in Mexico and grown for 3 yr in Colorado. For each of these scenarios the probability of the Enola haplotype was calculated on the basis of the frequency of the individual fragments in the populations of origin (see Materials and Methods; Table 3). Calculations were made easier because only a single fragment profile was identified for Enola (Fig. 3). As expected, the least probable scenario was the one in which the Enola fragment combination resulted from a cross between Andean and Mesoamerican genotypes (regardless of their seed color) represented in our sample (probability of 1×10^{-18}; Table 3). The scenario with the highest probability represented selection without hybridization within cultivar Azufrado Peruano 87 (3×10^{-1}). The three other scenarios had intermediate probabilities.

Comparison of Leaf Color among Yellow-Seeded Cultivars

Leaf color was examined because the statement of distinctness included in the Exhibit B (Statement of Distinctness) of the PVP certificate states that Enola most closely resembles the cultivar Azufrado Pimono 78 in a range of traits but differs from it in regards to leaf color, with Enola having lighter-colored leaves. A greenhouse experiment was conducted to compare the leaf color of a range of yellow-seeded cultivars grown under uniform conditions. There were no significant differences among replicates within cultivars. L was significant among cultivars ($P = 0.0419$), whereas Hue angle was not significant ($P = 0.3643$). There were highly significant differences among cultivars for Chroma ($P = 0.0020$). Comparisons of means showed no significant differences among cultivars for L and Hue angle. For Chroma, the only differences observed were between one of the sources of Azufrado Peruano 87 (Entry 46 in Table 1: 33.30), on the one hand, and Enola (27.35) and Azufrado Regional 87 from two sources (28.45, 28.77), on the other hand. For a second source of Azufrado Peruano 87 (Entry 49 in Table 1: 31.62), there were no significant differences with other cultivars, including Enola. Although Azufrado Pimono 78, the comparison cultivar included for distinctness in the PVP certificate, had darker green leaves (30.99) than Enola, the difference between the two cultivars was not significant in this experiment.

DISCUSSION

Choice of Markers for Fingerprinting

To conduct a fingerprinting experiment to determine the genetic relatedness among genotypes, three important elements need to be taken into account: the type of markers, the sample of genotypes, and the statistical treatment of the fingerprinting data. Fingerprinting markers should ideally have a high level of polymorphism, be numerous, and distributed throughout the genome. Microsatellite markers are the type of marker that best fits this description. However, few markers have yet been isolated and mapped in common bean (Métais et al., 2002; Yu et al., 1999, 2000; Blair et al., 2003). AFLPs (Vos et al., 1995) are an alternative to microsatellites. Among molecular markers (Jones et al., 1997; Powell et al., 1996), amplified fragment length polymorphisms (Vos et al., 1995) are advantageous because they reveal a high number of reproducible markers, thus, increasing the probability of identifying polymorphic markers even among closely related genotypes, including in common bean (Beebe et al., 2001; Tohme et al., 1996) and other crop species as well (Barcaccia et al., 1999; Caicedo et al., 1999; Coulibaly et al., 2002; Hongtrakul et al., 1997; Lombard et al., 2000; Mace et al., 1999; Mackill et al., 1996; Maughan et al., 1996; Roa et al., 1997; Xu and Sun, 2001). The low level of polymorphism of individual AFLP fragments is compensated by the large number of fragments revealed by each primer pair. In our study, each primer pair revealed around 70 fragments, of which 10 to 30% were polymorphic. This level of polymorphism is much lower than that found for *Eco*RI/*Mse*I primers in tall fescue, *Festuca arundinacea* Schreb (57%; Mian et al., 2002), the tropical tree *Pterocarpus officinalis* Jacq. (68%; Rivera-Ocasio et al., 2002), the Ethiopian cereals *Eragrostis* spp. (58%; Ayele and Nguyen, 2000), and common bean (over 90%; Tohme et al., 1996), but comparable to that observed in rice, *Oryza sativa* L. (28%; Mackill et al., 1996) and soybean, *Glycine max* (L.) Merr. (17–31%; Maughan et al., 1996). The discrepancy in polymorphism level between the results of Tohme et al. (1996) and these results may be attributed to the type of material. Tohme et al. (1996) analyzed wild beans, whereas this study was focused on domesticated beans, which have been subjected to a bottleneck of genetic diversity during and after domestication (Gepts, 1988; Gepts et al., 1986; Sonnante et al., 1994).

Most of the studies using AFLP markers use *Eco*RI and *Mse*I as restriction enzymes. In this research, both *Eco*RI/*Mse*I and *Pst*I/*Mse*I primer combinations were used on the same set of plant materials. The advantage of using *Pst*I/*Mse*I primer combinations arose mainly from a nearly 2.5-fold higher frequency of polymorphism over *Eco*RI/*Mse*I combinations. The *Eco*RI and *Pst*I enzymes sample different regions of the genome. The *Pst*I enzyme is methylation-sensitive and cuts principally in unmethylated regions of the genome, containing expressed and mainly single-copy genes. *Eco*RI, in contrast, is methylation-insensitive and cuts DNA throughout the genome. A similar observation was made for two restriction fragment length polymorphism (RFLP) clone libraries made after digestion of genomic bean DNA with *Eco*RI/*Bam*HI and *Pst*I. Digestion with the latter enzyme gave a higher frequency of single copy, polymorphic RFLP probes compared to digestion with the former enzymes (Nodari et al., 1992).

Results of the principal component analysis revealed overall patterns of genetic diversity similar to those observed previously with other markers, such as allozymes, RFLPs, and random amplified polymorphic DNA (RAPDs), and phenotypic traits (Gepts, 1998; Singh et al., 1991a). This increased our confidence that the AFLP approach would classify bean genotypes according to well-established genetic relationships. In addition, the AFLP analysis showed that Peruano-type yellow beans represent a distinct group, emphasizing their uniqueness even within the Andean gene pool.

Choice of Bean Cultivars for Analysis

The sample of common bean accessions analyzed in this study comprised two subsamples. A first subsample included a set of landrace accessions representing the six major races identified by Singh et al. (1991a). The other subsample included accessions with a yellow seed coat color similar to that of Enola (see Materials and Methods). The goal of establishing such a sample was to determine the most likely origin of Enola. This cultivar had to be compared to other yellow-seeded materials especially from Mexico as the patent description and PVP certificate both stated that this cultivar had been introduced from that country. Yellow beans from Mexico could have three possible origins: (i) the Mesoamerican gene pool, (ii) the Andean gene pool, and (iii) hybridization between these two gene pools. Furthermore, in evaluating probabilities of a match between fingerprints of yellow beans, one also needs to consider the same probabilities for more distantly related genotypes as a control.

Novelty (or the lack of it) is determined here by genetic relationships as defined by molecular markers. Previous research by Bassett et al. (2002) has shown already that the yellow color of Enola is controlled by the same gene combination present in Peruano class of Mexico. However, it is possible that, through breeding, the same yellow color could have been obtained from different parents with complementary genes for yellow seed color or with different genes or alleles for that color, altogether. Because of genetic redundancy, different gene combinations may lead to the same phenotype, leading one to erroneously infer a genetic identity. DNA fingerprinting of individual cultivars could help distinguish among these hypotheses about the origin of the yellow seed color. A close fingerprinting relationship or a complete match in the fingerprint would indicate that Enola is directly derived from Mexican yellow-seeded cultivars. In contrast, differences in fingerprinting between Enola and other cultivars would suggest a more distant relationship as would arise, for example, through breeding by hybridization.

Relationship of Current Data with the Enola Patent and PVP Certificate

Both the principal coordinate analysis of genetic diversity in our sample of common bean (Exp. 1) and the fingerprinting of the three cultivars (Enola, Azufrado Peruano 87, and Mayocoba; Exp. 2) show that Enola is most closely related to Azufrado Peruano 87 in our sample of yellow-seeded beans. Calculations of the probability of matching AFLP fingerprints showed that the most likely origin of Enola is by selection within pre-existing Mexican Peruano-type cultivars. This finding is consistent with the history of this genotype as outlined in the Enola patent and Appendix A of the PVP certificate. Both documents explain that Enola was obtained by selection of yellow seeds within a bean population heterogeneous for seed color, obtained in Mexico in 1994. Further presumed selection for yellow seed color, growth habit and height, flower and pod color, and leaf shape and size took place in 1995 and 1996 (Appendix A of the PVP Certificate). The uniformity of the AFLP banding pattern suggests that the sample submitted to the ATCC resulted from single seed selection during several generations before submission of the required seed sample to the ATCC.

In summary, we have determined the most likely origin of the cultivar Enola. The near to complete identity with pre-existing Mexican Peruano-type cultivars (present data) and the identity of the yellow seed color genotype with that of existing yellow bean cultivars (Bassett et al., 2002) raise questions about the rationales for the award of a utility patent and a PVP certificate for Enola. These questions are beyond the scope of this article.

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