Analysis of Electrophoresis Isozyme Patterns Using a Microcomputer-based Imaging System

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Abstract. A microcomputer-based image processing system was used to simplify the large number of visual comparisons required to identify various *Corylus* spp., cultivars, and clonal accessions using polyacrylamide gel electrophoresis isozyme patterns. Photographs of gels stained for peroxidase, acid phosphatase, and phenol oxidase were digitally captured and selected lanes were enhanced and scanned. The scan data were analyzed to locate bands and normalize their position to that of standards. Such data were plotted and a computer-generated isozyme pattern was displayed. Compressed image data were then stored in a database for subsequent automated isozyme pattern comparisons. Photographic records that were previously used in published reports were reevaluated with the computerized system. Species, cultivars, or clones that were characterized in visual evaluations were similarly characterized using the computer method. Computer evaluations usually identified more bands. Band positions were only rarely different and probably resulted from better normalization relative to standard bands when using the computerized procedure.

The National Clonal Germplasm Repository (NCGR), system collects, maintains, identifies, and characterizes clones of selected crop genera. As collections grow, identification and classification of new entries become more complex. For example, some genera maintained at the NCGR in Corvallis, Ore., already have close to 2,000 accessions (Jahn and Westwood, 1982). Thus, there is clearly a need for exact clonal identification procedures that can efficiently classify germplasm.

Biochemical identification of plant species and cultivars has received increased attention from plant breeders, the nursery industry, growers, and U.S. trade officials (Santamour and Demuth, 1980; Wolfe, 1976). In our work for NCGR, isozyme patterns of stem extracts of various species were resolved in all seasons (Ahmed et al., 1987; Menendez and Daley, 1986), and the patterns were constant for each clone (Ahmed et al., 1987; Menendez and Daley, 1986; Menendez et

al., 1986) regardless of plant age (Menendez and Daley, 1986). Experienced personnel can visually compare photographic records (PR) of isozyme patterns obtained by electrophoresis and consistently identify various clones. However, differences are often subtle, and considerable skill and experience are required. It is highly desirable that these comparisons be conducted by more objective means. Another limitation of visual inspection is the need to constantly compare the PR of specific accessions against all available PRs. Such comparisons with many accessions are difficult, especially when up to four isozyme patterns maybe required for identification.

The recent availability of relatively low-cost image capture and processing hardware for microcomputers permits the design and implementation of a system for enhancing, condensing, classifying, and identifying isozyme patterns. Condensed output can be placed in database software for sorting, classification, and identification of isozyme patterns by individuals without sophisticated electrophoretic interpretation skills. This paper describes the utility of such a system.

Plant material was obtained from the NCGR, Corvallis, *Corylus* collection. Protein extraction was conducted as described by Menendez and Daley (1986). One-year-old shoots (except when otherwise indicated) were stored at -30C before extraction since such storage is known to induce polyphenol oxidase (EC 1.10.3.2) activity (Daley et al., 1987). Electrophoresis was performed as previously described (Menendez and Daley, 1986) except that the room was at 3C. Staining for peroxidase (EC 1.11.1.7) and acid phosphatase (EC 3.1.3.2) was conducted as

described by Shaw and Prasad (1970) and phenol oxidase (EC 1.14.18.1) following Hare (1970). Enzyme substrates were: beta-naphthyl acid phosphate for acid phosphatase; 3-amino-9-ethyl carbazole for peroxidase; and catechol:p-phenylenediamine for phenol oxidase. A poorly defined region in the cathodic, low-density part of the gel stained lightly with all stains. This problem was attributed to the formation of enzyme polymers during extraction and was not useful for identification.

Gels were photographed with positive-negative film (Type 55, Polaroid, Cambridge, Mass.). These photographs were digitized and stored on a disk using a microcomputer-based image processing system. A JVC CCD TK-870U video camera with JVC HZ-C611AF(U) lens (JVC Co. of America, Elmwood Park, N. J.) was used to scan the photograph negatives. The video camera output was input to an AT&T Truevision (TARGA-24) image capture, digitizing, and display adapter with 512 × 512 pixel resolution and 256 levels of intensity for each of the red, green, and blue pixel components (AT&T Electronic Photography and Imaging Center, Indianapolis, Ind.). This adapter was installed in an IBM-AT microcomputer with 100 megabytes of fixed disk storage and 1.5 megabytes of random access memory. Gel images were recalled from disk and the inverse of pixel intensity (to produce a positive image) was displayed on a high resolution color monitor (SONY Trinitron CPD-1201, SONY Corp., Fujisawa, Japan). The software was written in C (Microsoft C, Version 5.1, Microsoft Corp., Redmond, Wash.) in menu-driven modular form.

Lanes to be scanned in the displayed stained gel images were interactively defined using a mouse to specify screen coordinates. The lane(s) of interest were then enhanced using a linear contrast stretch (Fig. 1). Digital filtering across the lanes (Elder and Southern, 1987a) was used to reduce noise in the scan. Scan data were collected across the selected lanes of the enhanced gel image and then smoothed using a simplified least-squares procedure (Savitzky and Golay, 1964) (Fig. 1). The scan data consist of average pixel intensity level (0 to 255) for the selected lanes at ≈ 500 intervals along the length of a lane, and were stored on disk for subsequent analysis. These results were normalized to the standard for each gel and to the standard for the series. The peaks in the scan data were found by applying a peak-finding routine using the first and second derivatives of the scan data similar to the method described by Elder and Southern (1987b). The isozyme pattern was then reconstructed from the normalized scan data, displaying located bands (Fig. 1, bottom). In this example, all bands were assigned the same intensity, and widths were defined as one-half the distance between the beginning and midpoints plus one-half the distance between mid- and end points of the peaks. Other criteria, such as midpoint plus and minus some constant, could work equally well. The sensitivity of this routine can be interactively adjusted. Increasing sen-

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Fig. 1. Electrophoretic gel enhancement using the computer gel analysis system. The phenol oxidase isozyme pattern from a filbert (*Corylus*) stem is shown. (top) Comparison of original isozyme pattern (left) and enhanced pattern (right). (bottom) Reconstructed gel pattern for lane 5 with scan of enhanced pattern displayed above and smoothed scan displayed below.

Fig. 2. Images of peroxidase isozyme patterns from *Corylus* spp. (top) Lane one was enhanced and scanned. The resulting computer-regenerated isozyme pattern is shown superimposed over lane 2 and the scan is plotted over the image. Lanes 3 and 4 are other *Corylus* spp. and are not enhanced. (bottom) The same gel processed as above, except with increased peak-finding sensitivity. Additional bands detected include: (a,b) visible in the enhanced lane 1; (c) visible as a peak in the plotted scan; and (d) probably spurious.

sitivity usually revealed bands that were difficult to resolve, but also usually recorded spurious peaks in the scan data. In the analysis shown in Fig. 2, four additional bands were recorded, two of which can be seen in the enhanced gel image, one of which can be seen as a peak in the scan plot, and one of which is probably spurious. In cases of bands that are particularly difficult to resolve, a more interactive peak-finding routine was used. The scan data were displayed over the enhanced gel image and a mouse was used to locate each peak (Fig. 3). In either case, the band locations given as beginning, mid- and end points of peaks in the scan data as well as relative densities were stored on disk.

Normalized, condensed gel data can be read into commercially available database software. In our application, dBase III+ (Ashton-Tate Torrance, Calif.) was used with a database structure and program that allows sorting and comparisons of up to 25 band positions that can be evaluated for both presence and intensity. Isozyme patterns obtained from plants of an unknown source can be matched with known patterns from an isozyme pattern database with minimal effort. Near matches, common bands, and other quantifiable similarities and differences can also be obtained.

For more rigorous interpretation of gel patterns, additional analysis of derivatives of scan data can be used to find peaks with ill-defined summits. This additional information can be added to the database.

To test the utility of the system, the acid phosphatase, peroxidase, and phenol oxidase isozyme patterns from 26 arbitrarily selected filbert (*Corylus*) species, cultivars, or clonal accessions were processed and stored in a database. Similar banding patterns were assigned to categories and these groupings were compared to published patterns (Ahmed et al., 1987) for the same species, cultivars, and clones. Each computer-derived pattern was used to select the closest match (highest number of similar bands) among the published patterns for each isozyme. Six independently obtained high quality patterns for *C. avellana* 'Barcelona' were also added to the database. When the computer-derived pattern from any one of these gels was used as the search pattern, all other occurrences of 'Barcelona' in the database were selected as matches.

In Table 1 we compare the number of bands found by the computer method with those found by Ahmed et al. (1987). The computer procedure identified more bands in 86% of the cases. (Not all bands were considered in the visual method.) When using the computer-assisted method, it is less cumbersome to consider a larger number of bands than in visual evaluations. Bands that were identified visually were almost always identified with the computer procedure. Fewer or different bands were only rarely identified.

A comparison of the patterns found by the computer method with those found by Ahmed et al. (1987) is displayed in Table 2. Each unique pattern was assigned to a different group. The left three columns in Table 2 for

Table 1. Differences in number of bands published previously and located by the computer method.

Species, cultivar, or clonal accession (NCGR number)	No. published bands ²				No. computer-found bands ²				Different ² (%)			
	AP	P	PPO	Total	AP	P	PPO	Total	AP	P	PPO	Total
<i>Corylus avellana</i>												
Barcelona (36)	7	6	4	17	8	10	6	24	114	167	150	141
<i>C. avellana</i> Brixnut (26)	6	4	5	15	7	---	8	---	117	---	160	---
<i>C. avellana</i> Casina (28)	6	5	5	16	7	9	---	---	117	180	---	---
<i>C. avellana</i> Crossal de Constanti (75)	6	4	5	15	7	---	10	---	117	---	200	---
<i>C. avellana</i> Fitzgerald (27)	7	2	2	11	8	8	---	---	114	400	---	---
<i>C. avellana</i> var. <i>fuscorubra</i> (39)	8	6	5	19	8	12	8	28	100	200	160	147
<i>C. avellana</i> Gem (23)	6	5	6	17	8	12	10	30	133	240	167	176
<i>C. avellana</i> Gironenc (44)	7	5	6	18	6	9	10	25	86	180	167	139
<i>C. avellana</i> Italian Red (30)	6	5	6	17	6	13	10	29	100	260	167	171
<i>C. avellana</i> Kruse (25)	7	2	1	10	7	9	4	20	100	450	400	200
<i>C. avellana</i> var. <i>heterophylla</i> (159)	7	3	4	14	8	10	---	---	114	333	---	---
<i>C. avellana</i> Montebello (17)	7	5	5	17	8	12	9	29	114	240	180	171
<i>C. avellana</i> Mortarella (51)	7	6	6	19	8	13	---	---	114	217	---	---
<i>C. avellana</i> Negret (8)	7	4	4	15	8	11	---	---	114	275	---	---
<i>C. avellana</i> Neue Riesennuss (10)	6	6	6	18	7	---	6	---	117	---	100	---
<i>C. avellana</i> OSU 14-84 (52)	7	4	2	13	8	10	---	---	114	250	---	---
<i>C. avellana</i> OSU 54-50 (93)	6	5	3	14	7	13	8	28	117	260	267	200
<i>C. avellana</i> Segorbe (20)	7	5	6	18	8	13	11	32	114	260	183	178
<i>C. avellana</i> Tombul Ghiaghli (55)	7	6	6	19	6	10	---	---	86	167	---	---
<i>C. avellana</i> Tonda Bianca (21)	7	5	5	17	9	12	---	---	129	240	---	---
<i>C. avellana</i> Woodford (12)	6	4	2	12	7	11	---	---	117	275	---	---
<i>C. columna</i> var. <i>chinensis</i> (19)	7	3	3	13	8	10	---	---	114	333	---	---
<i>C. heterophylla</i> (18)	7	3	4	14	7	10	7	24	100	333	175	171
<i>C. sp.</i> BR-4-1 (136)	6	4	4	14	7	---	10	---	117	---	250	---
<i>C. sp.</i> USOR 5-70 (125)	7	3	4	14	8	9	9	26	114	300	225	186
<i>C. sp.</i> USOR 6-73 (144)	7	5	4	16	7	9	10	26	100	180	250	163
Mean									111	261	200	170

²AP, acid phosphatase; P, peroxidase; PPO, phenol oxidase.

--- = gel for this species, cultivar, or clone not available.

Fig. 3. A screen illustrating the interactive method of peak location. With the scan displayed on the enhanced gel image, it becomes an easy task to locate peaks using a mouse-controlled cursor.

Fig. 4. Illustration of cases where the computer gel analysis system found a pattern different from published patterns. (left) From top to bottom, the published pattern, the computer-normalized, regenerated pattern, the enhanced image, and the original acid phosphatase image for *C. avellana* Gem (23). (right) Published (top two patterns) and computer-regenerated (bottom two patterns) peroxidase patterns from species *C. heterophylla* (18) and *C. avellana* var. *heterophylla* (159) whose patterns differ in the previous reports but are remarkably similar after computer evaluation.

acid phosphatase, peroxidase, and phenol oxidase are pattern groups assigned by the computer method. The three columns on the right are pattern numbers used by Ahmed et al. (1987). Even though the computer procedure identified more bands, categorizations are similar. Subscripted numbers in the best-match columns are the published pattern number where disagreement occurred. For example, Ahmed et al. (1987) placed NCGR accessions 17, 23, and 20 in pattern category 21 for acid phosphatase, but the computer method assigned these three accessions to our category A, which corresponds to the Ahmed et al. (1987) category 1. In 91% of the cases, the same bands listed in the pre-

vious publication were found in the appropriate position when using the computer procedure. In 80% of the cases, patterns designated in the Ahmed et al. (1987) paper were the closest match. In the remaining 20%, better matches were found. Half of these better matches are accounted for by using bands not observed by the original researchers.

Table 3 lists the accessions where patterns differed from those published (Ahmed et al., 1987) and the reasons they differed. Most differences occurred in the acid phosphatase patterns and were mainly due to the computer method detecting more bands. There were three instances in which fewer bands were detected and three instances of bands

located in different positions. In cases where position was altered, differences in standardization are a probable cause. Figure 4 (left) illustrates additional band detection for the case of *C. avellana* Gem. The computer-assisted analysis was able to resolve the two bands on the left that were recorded as a single band in the visual method. It also detected the second band from the right, which was not detected in the visual method. Figure 4 (right) reveals differences in both band number and location. Since all 10 bands of the regenerated patterns match, it is likely that position differences reported in the visual three-peak categorization are due to standardization error and the two species should

Table 2. A comparison of published (Ahmed et al., 1987) and computer-generated isozyme banding patterns for acid phosphatase (AP), peroxidase (P), and phenol oxidase (PPO) found in 26 *Corylus* spp., cultivars, and clonal accessions.

Species, cultivar, or clonal accession (NCGR number)	Enzyme staining pattern					
	Pattern categories assigned for computer method ^a			Best-matched pattern numbers from published work ^a		
	AP	P	PPO	AP	P	PPO
<i>Corylus avellana</i> Barcelona (36)	A	A	A	1	1	1
<i>C. avellana</i> Montebello (17)	A	B	B	1 ₂₁	42	39
<i>C. avellana</i> Mortarella (51)	A	C	---	1	26	---
<i>C. avellana</i> Negret (8)	A	D	---	1	22	---
<i>C. avellana</i> OSU 14-84 (52)	A	E	---	1	25	---
<i>C. avellana</i> Gem (23)	A	F	C	1 ₂₁	43	40
<i>C. avellana</i> Segorbe (20)	A	G	D	1 ₂₁	40	37
<i>C. avellana</i> var. <i>heterophylla</i> (159)	B	H	---	4	30	---
<i>C. sp.</i> USOR 5-70 (125)	B	I	E	4	4	4
<i>C. columna</i> var. <i>chinensis</i> (19)	J	J	---	4	23	---
<i>C. heterophylla</i> (18)	C	H	F	5	30 ₅	6 ₄
<i>C. sp.</i> BR-4-1 (136)	C	---	G	5 ₁₄	---	18
<i>C. sp.</i> USOR 6-73 (144)	C	K	H	5	9 ₆	8
<i>C. avellana</i> Woodford (12)	D	L	---	2	21 ₂₅	---
<i>C. avellana</i> Casina (28)	D	M	---	2	24	---
<i>C. avellana</i> Tombul Ghiaghli (55)	E	N	---	25 ₂₀	8 ₂₆	---
<i>C. avellana</i> Italian Red (30)	E	O	I	25	45	44
<i>C. avellana</i> Brixnut (26)	F	---	J	14	---	15
<i>C. avellana</i> Crossal de Constanti (75)	F	---	K	14	---	14
<i>C. avellana</i> Fitzgerald (27)	G	P	---	31 ₁	29	---
<i>C. avellana</i> var. <i>fuscoburba</i> (39)	H	Q	L	27	47	46
<i>C. avellana</i> Neue Riesennuss (10)	I	---	M	13	---	13
<i>C. avellana</i> Tonda Bianca (21)	J	R	---	19 ₁	28	---
<i>C. avellana</i> Kruse (25)	K	S	N	26	32	45
<i>C. avellana</i> Gironenc (44)	L	T	O	6	6	5
<i>C. avellana</i> OSU 54-50 (93)	M	U	P	18 ₂	2	2

^aSubscript numbers are the published pattern number where disagreement occurred.

--- = gel for this cultivar, species, or clone not available.

Table 3. Instances of disagreement when comparing patterns found by computer analysis with published patterns.

Species, cultivar, or clonal accession (NCGR number)	Band(s) ^a								
	Additional found			Fewer found			In different position		
	AP	P	PPO	AP	P	PPO	AP	P	PPO
<i>C. avellana</i> Fitzgerald (27)	X								
<i>C. avellana</i> Gem (23)	X								
<i>C. avellana</i> Montebello (17)	X								
<i>C. avellana</i> OSU 54-50 (93)	X								
<i>C. avellana</i> Segorbe (20)	X								
<i>C. avellana</i> Tombul Ghiaghli (55)				X	X				
<i>C. avellana</i> Tonda Bianca (21)	X								
<i>C. avellana</i> Woodford (12)		X							
<i>C. heterophylla</i> (18)						X		X	
<i>C. sp.</i> BR-4-1 (136)							X		
<i>C. sp.</i> USOR 6-73 (144)								X	

^aAP, acid phosphatase; P, peroxidase; PPO, phenol oxidase.

be in similar categories for this enzyme.

The method of gel analysis described in this paper appears to be a cost-effective, accurate, and time-saving tool for the analysis of gels from polyacrylamide electrophoresis, the determination of isozyme patterns, and the comparison of these patterns. In our comparison with previously published isozyme patterns for a set of *Corylus* spp. and cultivars, the computer method was successfully used to characterize the species and cultivars. When compared with the visual method, persons unskilled in interpreting gel patterns can perform the characterization task with equal or better effectiveness. Similar results can be obtained regardless of the evaluator, thus biases in interpretation are reduced.

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