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# Fingerprinting Apple Cultivars by Electrophoretic Isozyme Banding Patterns[1]

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## Abstract

Anionic electrophoretic isozyme patterns of peroxidase, esterase and acid phosphatase and cationic peroxidase isozyme patterns from shoot bark protein extracts were used to identify clonal apple scion cultivars. Each of the 21 cultivars included in this study developed a unique combination of isozyme patterns which allowed it to be distinguished from the others. Sports within cultivars exhibited identical patterns of enzymes, with the exception of 'Wijcik', a natural compact mutant of 'McIntosh' which could be distinguished from the latter, although it was indistinguishable from the cultivar 'Spartan'. Isozyme patterns remained constant when samples were taken from wood of different ages, at several times of the year and with trees growing in different locations and on different rootstocks.

**Index words:** cultivar identification, *Malus domestica*, protein analysis

## Introduction

Cultivar identification has recently gained considerable attention because of its financial and legal implications (5). Currently, classification of fruit cultivars is based on morphological characteristics (1, 4, 11, 18). These classifications are based chiefly on flowers and fruit and other easily observed, often subjective, characteristics that may vary widely with the environment. Since morphological classifications are based on descriptions of adult specimens with flowers and fruits, they are largely useless for identifying immature or juvenile individuals.

Chemical markers have proven to be extremely useful for the identification of many economically important crops. Isozyme polymorphism (genetic variance) has been researched in several horticultural plants for the purpose of cultivar identification, i.e. strawberry (2), 'Kentucky' blue grass (16), grapes (17), and roses (6), among others. Apples and other clonally propagated temperate fruits are well suited for isozyme analysis because there should be minimal genetic variability within the individuals of a cultivar (13, 15).

In our previous work (7, 8, 9), we demonstrated that: a) isozyme diversity could be used for positive identification of apple rootstock clones, and b) for the enzymes reported in the present study, banding patterns were not altered by sampling time or environment and were constant within individuals of the same clone.

The objective of this study was to test the applicability of techniques previously developed (for the study of rootstock clones) in our laboratory (7, 8, 9) for the identification of apple cultivars used for fruit production (scion cultivars) and their natural mutations (sports), and to determine the effect of age of sampled wood, rootstock, sampling time, and growing location on banding patterns.

## Materials and Methods

One-year-old scion shoots were obtained from commercial nurseries in the Yakima and Wenatchee areas of WA, from the Washington State University horticultural farm at Pullman, and the Washington State University Tree Fruit Research Center at Wenatchee, WA. Cultivars and sports used are as listed in Table 1. All those available in Pullman were sequentially sampled 4 times throughout the year (Feb-

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ruary, May, August, November) using 3 replications at each sampling time. The same trees used were for the entire sampling sequence.

Three cultivars and/or sports ('Goldspur', 'Top Red', and 'Imperial Red McIntosh'), each on 3 rootstocks (M 26, MM 106, and MM 111), were sampled to evaluate possible rootstock effects on banding patterns. These cultivars were also used to compare isozyme patterns from wood of 1-, 2-, and 3-year-old shoots, using 4 trees of each cultivar sampled in February and August.

To test the effect of growing location, 6 cultivars and/or sports ('Wellspur', 'Red Chief', 'Red King', 'Starkrimson', 'Imperial Red McIntosh', 'Spur Winter Banana', and 'Golden Delicious') were sampled in February and August at Pull-

**Table 1. Apple cultivars used for isozyme analysis of extracts from shoot bark tissue.**

| | | Source | | |
|---|---|---|---|---|
| **Cultivar** | **Sport** | **WSU[z]** | **WSU[y]** | **Commercial Nurseries[x]** |
| Cortland | | + | | |
| Cox's Orange Pippin | | + | | |
| Empire | | + | | |
| Golden Delicious | | + | | |
| | Goldspur (Sundale) | + | + | + |
| | Criterion | | | + |
| | Firmgold | | | + |
| | Earligold | + | | + |
| Granny Smith | | + | | |
| | Spur Granny Smith | + | + | + |
| Gravenstein | | + | | |
| Idared | | + | | |
| Jonagold | | + | | |
| Jonamac | | + | | |
| Law Spur Rome Beauty | | + | | |
| Lodi | | + | | |
| McIntosh | | + | | |
| | Imperial | + | | + |
| | Roger's | | | + |
| | Wijcik | + | | |
| Mutsu | | + | | |
| Northern Spy | | + | | |
| Priscilla | | + | | |
| Rhode Island Greening | | + | | |
| Spartan | | + | | |
| Starking Delicious | | + | | |
| | Ace | | | + |
| | Aomori | + | | |
| | Apex | + | | + |
| | Atwood | | | + |
| | BM-62 | | | + |
| | Brite and Early | | | + |
| | Cascade | | | + |
| | Classic | | | + |
| | Early Brite | + | | + |
| | Early Red One | | | + |
| | Hi-Early | + | | + |
| | Oregon Spur II | + | | + |
| | Real McCoy | | | + |
| | Red Chief | + | | + |
| | Red King | + | | + |
| | Red Spur | + | | |
| | Ryan Red Improved | | | + |
| | Scarlett Spur | | | + |
| | Sharp Red | | | + |
| | Silver Spur | | | + |
| | Spur Ryan Red | | | + |
| | Starkrimson | + | + | + |
| | Top Red | + | + | + |
| | Top Spur | | | + |
| | Wellspur | + | | + |
| Tydeman's Red | | + | | |
| Winesap | | + | | |
| | Nured Winesap | | | + |
| Winter Banana | | + | | |
| | Spur W. Banana | + | | + |

[z]Pullman

[y]Tree Fruit Research Center, Wenatchee

[x]Wenatchee and Yakima areas



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man and at commercial nurseries in Yakima and Wenatchee. Winter samples from nurseries were from trees in cold storage.

In the above tests, 'Northern Spy' and Starking Delicious were run as reference in all gels.

For protein extraction, shoots were washed with deionized water, blotted dry, and the bark was peeled off and cut up with a razor blade. Six grams of bark and 24 ml of a 50mM Tris-HCl, pH 8.3 buffer containing 1mM $Na_2EDTA$ 3mM dithioerythritol, 5mM ascorbic acid, 3mM $Na_2S_2O_5$, 6mM diethyldithiocarbamate, 5mM phenyl-methyl sulfonyl fluoride, 14mM β-mercapto-ethanol, 0.1% Triton X-100 and 2g hydrated polyvinylpolypyrrolidone per g of tissue, were ground in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) 4 times for 30 sec at 0–1°C. The homogenate was centrifuged at 20,000 g for 20 min, and the supernatant was either divided in 0.5ml aliquots and frozen at −40°C or used immediately for electrophoresis.

For the separation of isozymes, 3 electrophoretic systems were used; System I, as described by Davis (3), with the following modifications: the electrode buffer was diluted 1:4 instead of 1:9; the separating gel was 5T5C-15T5C polyacrylamide linear gradient (linear gradient maker SG 105 S, Hoefer Scientific Instruments, San Francisco, CA.) instead of 7.5T5C; and both the separating and stacking gels contained 0.5% Triton X-100. System II consisted of 300mM Tris-HCl, pH 8.0, 5T5C-15T5C polyacrylamide gradient separating gel; 100mM Tris-$H_3PO_4$, pH 6.5, 4T5C stacking gel; the upper chamber buffer was 37mM Tris, 40mM glycine, pH 8.9, and the lower chamber buffer, 63mM Tris-HCl, ph 7.5. System III was the cathodic system of Reisfeld et al. (10), used with a linear gradient separating gel (5T 0.66-20T 0.66C), and modified according to the LKB laboratory manual (LKB Produkter AB, Bromma, Sweden). For System I, the LK 2001 (LKB Produkter, Bromma, Sweden), dual vertical slab electrophoresis unit was used. For

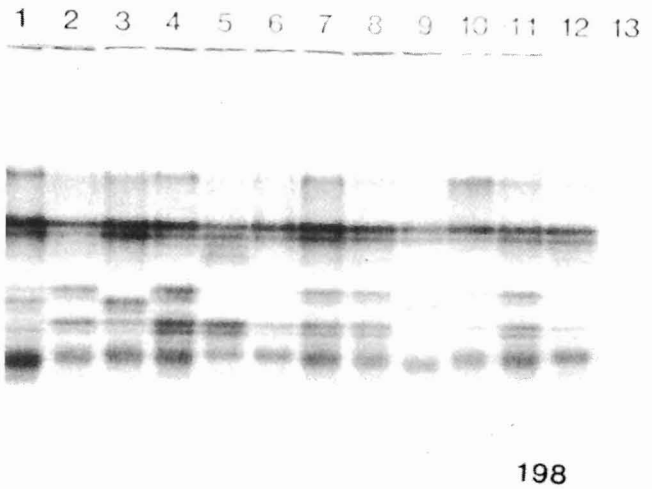


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**Fig. 1. Anionic peroxidase isozymes of clonal apple cultivars. From left to right: Northern Spy, Spur Winter Banana, Law Spur Rome, Goldspur (Sundale), Spartan, Starking Delicious, Criterion, Firmgold, Cox's Orange, Winter Banana, Earligold, Winesap.**

Systems II and III, the Model 220 (Bio-Rad Laboratories, Richmond, CA.), dual vertical slab unit was used. Gels were 1.5mm thick in all cases.

Samples were mixed with 60% glycerol and 0.1% bromophenol blue and underlayered with a micropipette. Loading was adjusted in each case according to activity and clarity of the bands, based on preliminary runs. Electrophoresis was run in a cold room (1–2°C) at constant current (40mA for Systems I and II, 60mA for System III), with 300V limit for both, until the tracking dye reached 0.5cm from the bottom of the gel.

Three enzymes (chosen on the basis of preliminary work) were used for clone identification: 1) peroxidase, run in

**Table 2. Isozyme banding patterns[z] produced by enzyme systems used to distinguish apple cultivars. 'Northern Spy' was used as a comparative standard.**

| Pattern Combinations | Cultivar | Enzyme systems: Anionic peroxidase | Anionic esterase | Anionic acid phosphatase | Cationic peroxidase |
|---|---|---|---|---|---|
| I | Cortland | 1[z] | 10 | 2 | 1 |
| II | Cox's Orange Pippin | 1 | 9 | 1 | 1 |
| III | Delicious (Starking and its sports) | 2 | 2 | 2 | 1 |
| IV | Empire | 2 | 7 | 4 | 1 |
| V | Golden Delicious (and its sports) | 3 | 3 | 3 | 2 |
| VI | Granny Smith and Spur Granny Smith | 7 | 10 | 1 | 3 |
| VII | Gravenstein | 8 | 2 | 1 | 2 |
| VIII | Idared | 5 | 5 | 2 | 1 |
| IX | Jonagold | 4 | 4 | 3 | 1 |
| X | Jonamac | 4 | 5 | 3 | 1 |
| XI | Law Spur Rome Beauty | 8 | 9 | 2 | 1 |
| XII | Lodi | 6 | 9 | 2 | 1 |
| XIII | McIntosh (Imperial and Roger's sports) | 2 | 6 | 4 | 1 |
| XIV | Mutsu | 4 | 4 | 4 | 3 |
| XV | Northern Spy | 1 | 1 | 1 | 1 |
| XVI | Priscilla | 4 | 2 | 3 | 1 |
| XVII | Rhode Island Greening | 1 | 10 | 2 | 1 |
| XVIII | Spartan, Wijcik | 2 | 6 | 4 | 2 |
| XIX | Tydeman's Red | 8 | 2 | 2 | 1 |
| XX | Winesap and Nured Winesap | 2 | 8 | 2 | 1 |
| XXI | Winter Banana and Spur Winter Banana | 4 | 7 | 2 | 1 |

[z]Cultivars with the same pattern number within a column are not distinguishable with that enzyme.



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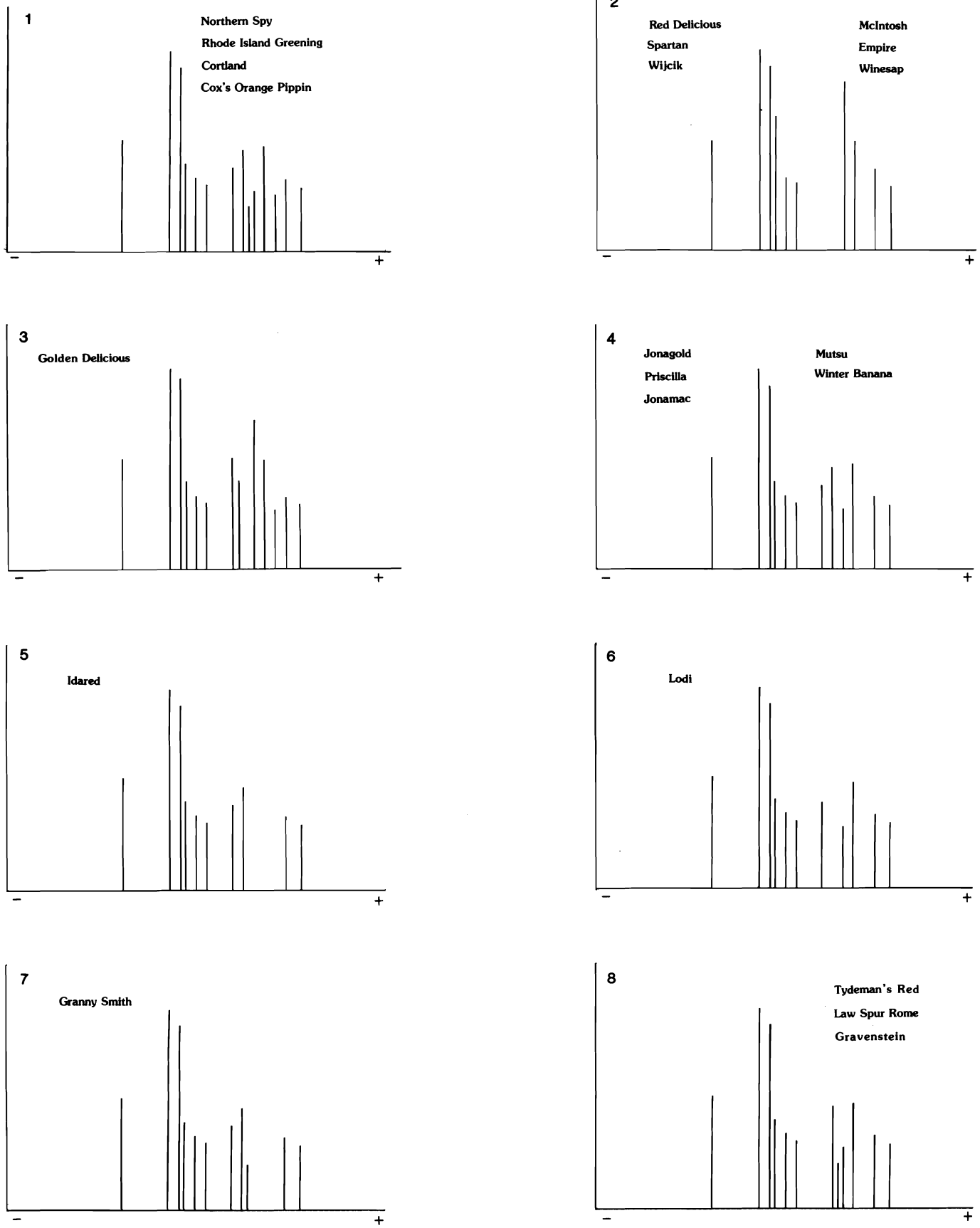


**Fig. 2.** Anionic peroxidase isozyme banding patterns of clonal apple cultivars as depicted by densitometric scanning. Vertical bars represent relative position of bands in the gels; height indicates band intensity. Numbers in the upper left-hand corner of each pattern refer to banding patterns listed in Table 2.

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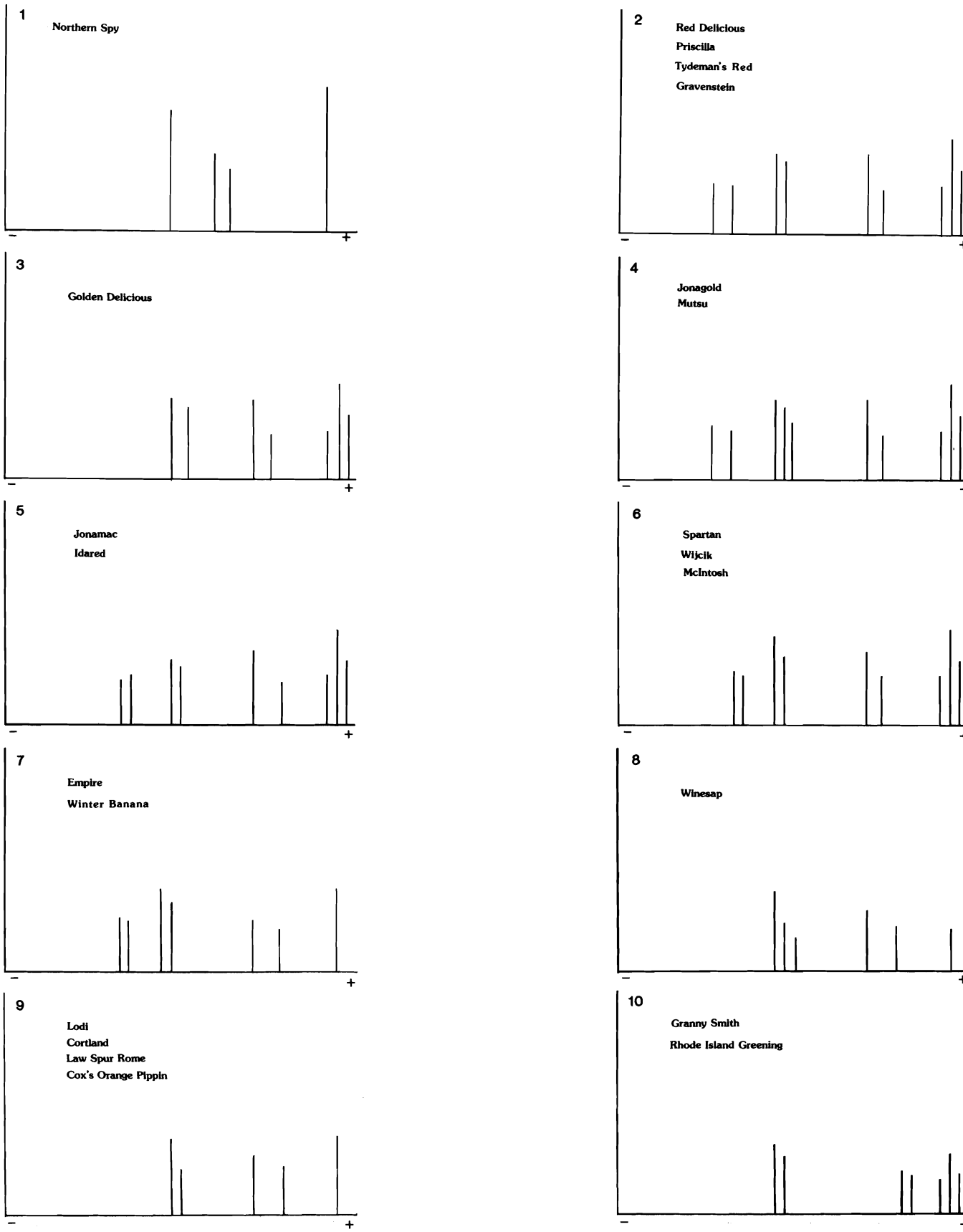


**Fig. 3.** **Anionic esterase isozymes banding patterns of clonal apple cultivars as depicted by densitometric scanning. Vertical bars represent relative position of bands in the gel; height indicates band intensity. Numbers in the upper left-hand corner of each pattern refer to banding patterns listed in Table 2.**

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Systems II and III and stained according to Shaw and Prasad (12); 2) esterase, run in System I without Triton X-100 and stained (12) using α-naphthyl acetate; 3) acid phosphatase, run in System I using Na β-naphthyl acid phosphate (14).

Upon staining and fixing, gels were photographed with Polaroid positive/negative film, Type 55 (Polaroid Corp., Cambridge, MA.), and the negatives were scanned at 540nm using a Cary 210 Spectrophotometer with gel scanning attachment. Peaks identifiable with the densitometer were transformed to vertical bars, indicating peak position and height. Peak position was adjusted using two reference bands to correct for slight inaccuracies caused by gel stretching or shrinking. Banding patterns observed for each enzyme and/or electrophoretic condition were then numbered and grouped accordingly.

## Results and Discussion

All cultivars tested (Table 1) were positively identified by the cross-examination of the isozyme banding patterns of 4 enzyme systems. Sports within the same cultivar, however, were indistinguishable (Fig. 1). The only exception was 'Wijcik', a natural compact mutant of 'McIntosh', which could be distinguished from the latter but appeared indistinguishable from 'Spartan'. Banding patterns obtained by densitometric scanning of each enzyme and cultivar are illustrated (Figs. 2–5) and the results are summarized (Table 2).

As with the clonal apple rootstock cultivars previously studied (9), isozymic diversity made identification of apple scion cultivars feasible. Sampling time, rootstock, location, and sample age (1-, 2-, or 3-year-old stems) had no effect on scion banding patterns (data omitted for brevity). Each enzyme, however, produced fewer patterns than previously found for clonal rootstock cultivars (9), indicating a more narrow genetic base.

One desirable objective, identification of sports within cultivars, could not be achieved by these methods. Even though some commercially named sports may in fact be genetically identical, the technique failed to distinguish between spur and non-spur types as well as between very early coloring sports and the original 'Starking Delicious'. The chimeral nature of most sports apparently did not affect the genetic base of the enzymes most commonly used for cultivar fingerprinting, or sampling was not selective enough to demonstrate a difference.

## Significance to the Nursery Industry

With the exception of distinguishing between sports within cultivars, the methods for fingerprinting apples described here can be used as an aid for the identification of apple nursery stock when the identity of a lot of trees is questioned. The procedure can be applied to young budded trees and can be used at any time of the year (during the growing season or when the trees are in storage after digging). Only

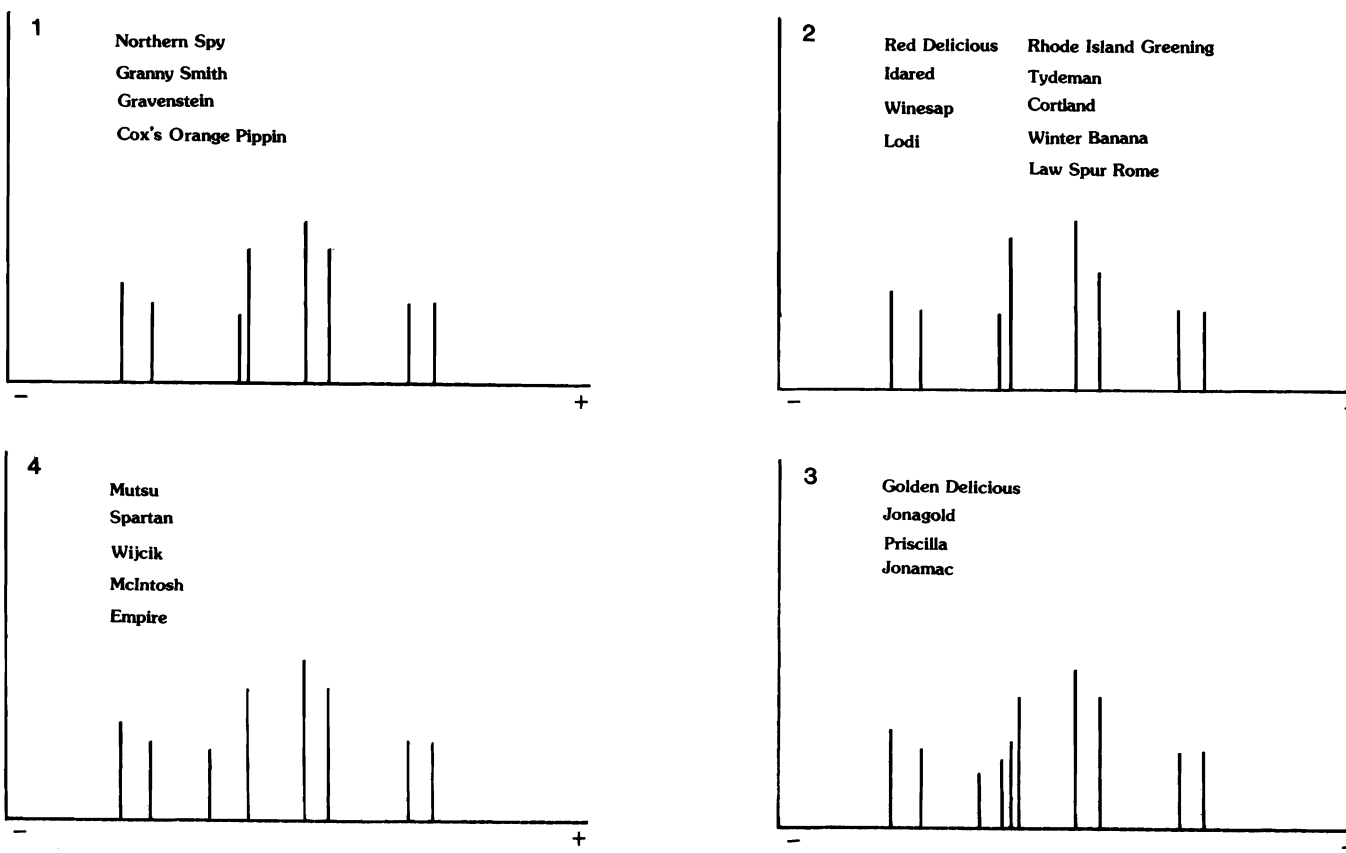


**Fig. 4. Anionic acid phosphatase isozyme banding patterns of clonal apple cultivars as depicted by densitometric scanning. Vertical bars represent relative position of bands in the gel; height indicates band intensity. Numbers in the upper left-hand corner of each pattern refer to banding patterns listed in Table 2.**

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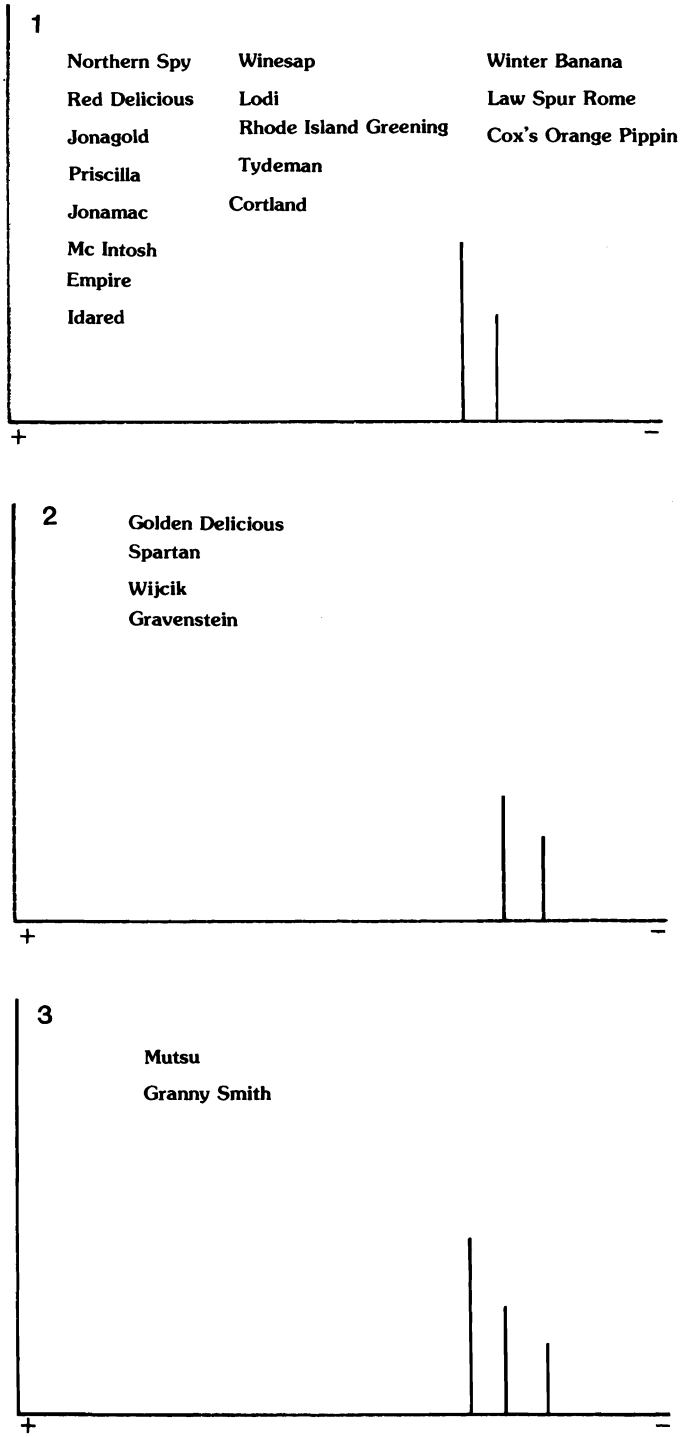


**Fig. 5. Cationic peroxidase isozyme banding patterns of clonal apple cultivars as depicted by densitometric scanning. Vertical bars represent relative position of bands in the gel; height indicates band intensity. Numbers in the upper left-hand corner of each pattern refer to banding patterns listed in Table 2.**

a small piece of stem is required. Also, these methods may assist in the advancement of breeding programs and may be useful in new cultivar descriptions and patent protection.

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