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# The relationships between *Prunus avium, Prunus mahaleb* and *Prunus fontanesiana*, studied by means of thin layer chromatography

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## INTRODUCTION

Biochemical comparisons have in the past often been used for the study of the relationships between various kinds of living organisms. The history of this branch of science has been amply dealt with, e.g. by Alston and Turner in their book (1963). However, with the introduction of more efficient analytical methods, such as chromatography, biochemical analyses have been taken into more widespread use for the elucidation of taxonomical problems.

It is certainly no exaggaration to say that especially the application of paper chromatography has been a very strong incitement for the use of biochemical taxonomy (cf., e.g., Bate-Smith 1962). Especially in the last few years a great many applications of paper chromatography are reported for chemotaxonomical purpose.

The substance most frequently used for tracing the relationships between different taxa or biotypes has been the rich variety of phenolic derivatives found in the leaves. They are most easily revealed by means of their UV-fluorescence. Their reliability for taxonomical purposes has been demonstrated by McClure and Alston (1966). Valuable information on their chemistry, genetics and systematic occurrence is found in the volume edited by Harborne (Harborne 1964).

Thin-layer chromatography has in many cases proved to be a useful refinement as compared with paper chromatography. There are, however, still very few examples of its use for taxonomical studies (cf. Brunsberg 1965; Grant and Whetter 1966).

In order to improve the efficiency of TLC and make it more suitable for serial analyses, such as are necessary in taxonomy when a number of different plants or taxa have to be dealt with, we have developed a special TLC system, which shall be here described in some detail, as it differs almost completely from the conventional systems. It has also been found to be particularly well suited for chemotaxonomy (cf. Jaworska and

Nybom 1966; also Dass and Nybom 1967, and Oldén and Nybom 1968).

This TLC-taxonomy has with good success been applied to a number of different objects:

— for differentiating maternal or parthenogenetically produced offspring from true hybrids in hybridization experiments;

— for tracing intermixtures and variety contamination in various plant breeding projects;

— for distinguishing varieties of, e.g. black-currants and raspberries, presenting a wide variation in the pattern of leaf phenolics, of flowers exhibiting a variation in flower colour (Muszyński 1964);

— for genetical experiments, studying the inheritance of anthocyanin pigments, e.g. in *Petunia* (Muszyński, in preparation);

— for clearing up or elucidating cases of reputed spontaneous hybrids, where the exact details as to parentage are not known for certain (cf. Jaworska and Nybom, Olden and Nybom and the present paper).

The present study is to some extent intended to present the possibilities of TLC for chemotaxonomy and also to show some lines along which these potentialities may be utilized in practice. The material used consists of the cherry type *Prunus fontanesiana* and its reputed parents, the sweet cherries, *Prunus avium*, and the St. Lucien cherry, *Prunus mahaleb*.

## MATERIAL AND METHODS

*Prunus mahaleb*, the St. Lucien cherry is in rather extensive use as a dwarfing root-stock for sweet cherries, *Prunus avium*. One condition, that limits this use is a partial, vegetative incompatibility between these two species. For this reason, hybrids between *Prunus mahaleb* and *Prunus avium* might be of interest. *Prunus fontanesiana* is an *avium*-like type which is supposed to be an old hybrid between the two species in question (cf. Rehder 1949). This does not seem to have been proved experimentally, however, therefore we decided to submit the case to a study by means of thin-layer chromatography.

For this purpose leaves of *Prunus avium* and of *Prunus mahaleb* were collected during summer and dried. Dry leaves of *Prunus fontanesiana* for comparison were kindly made available from the Kew Gardens England. Our thanks are due to the Balsgard cherry specialist, Mr. E. J. Oldén for placing this material at our disposal.

For chromatographic analysis leaf phenolics were extracted with acidified methanol. To 0.1 g of leaf powder in a glass tube 1 ml of 0.1%

Table 1

Fluorescence colour and spot surfaces in mm² of phenolic compounds in *Prunus* leaves after spraying with aluminium chloride and sodium hydroxide

| Spot number | Reagent and colour | Spot surface | | |
|---|---|---|---|---|
| | | *P. avium* | *P. fontanesiana* | *P. mahaleb* |
| 1 | $AlCl_3$; yellow | — | + | — |
| 2 | | — | + | — |
| 3 | | 75 | 60 | + |
| 4 | | 60 | 60 | 30 |
| 5 | | 60 | 80 | 30 |
| 6 | | 50 | 90 | 50 |
| 7 | | + | — | — |
| 8 | | — | + | + |
| 9 | | — | + | + |
| 10 | | + | — | — |
| 11 | | + | + | — |
| 12 | | + | + | — |
| 13 | | 90 | 90 | 130 |
| 14 | | — | 75 | 100 |
| 15 | | 40 | 75 | + |
| 16 | | 20 | 30 | — |
| 17 | | + | + | — |
| 18 | | + | — | — |
| 19 | | — | + | — |
| 20 | $AlCl_3$; green | + | — | — |
| 21 | | 30 | 25 | — |
| 22 | | 45 | 20 | — |
| 23 | | + | — | — |
| 24 | | + | + | — |
| 25 | | 15 | — | — |
| 26 | | 35 | + | — |
| 27 | | + | + | — |
| 28 | | + | + | — |
| 29 | $AlCl_3$ blue | 20 | 20 | — |
| 30 | $AlCl_3$; + NaOH; blue | + | + | — |
| 31 | | + | + | — |
| 32 | | + | + | — |
| 33 | | 40 | 20 | 20 |
| 34 | | + | + | + |
| 35 | $AlCl_3$+NaOH green | + | — | — |
| 36 | | + | — | — |
| 37 | | + | + | — |
| 38 | | + | 20 | + |
| 39 | | + | + | — |
| 40 | | + | + | — |
| 41 | | + | + | — |
| 42 | | + | + | + |

methanolic hydrochloric acid was added and the glass tubes were continuously shaken during 4 hours at room temperature. After centrifugation, the extract was chromatographed on cellulose coated glass plates, the cellulose powder MN 300 being used as adsorbent.

Two-dimensional thin layer chromatography was used in the following solvent systems:

1) 2% formic acid in water
2) 20 parts of normal amyl alcohol (AmOH): 12 parts of conc. acetic acid (HAc): 10 parts of water; it forms one phase only.

The chromatograms were analysed under UV-light, the fluorescence of phenolic substances being intensified by spraying the plates with 1% sodium hydroxide followed by 1% alluminium chloride, both dissolved in methanol. The first reagent especially intensifies the blue-white (or greenish) fluorescence of hydroxyaromatic acids, whereas alluminium chloride is more useful for the yellow fluorescing flavonoids. Sodium hydroxide produces a permanent effect, similar to that of fuming with ammonia used by some authors.

Several plates were made for each sample. The best plate from the same sample, i.e. with best resolution and distinctness, is used for marking and encircling all detectable spots. For marking spots of different colours a special system of hatching designed by Nybom (1967) was used. While encircling the spots it was tried to make their surfaces as proportional to the size and intensity of fluorescence as possible. When all detectable spots had been marked for all three taxa included in the study, they were copied over together on one clean plate, and thus a composite plate or master plate was obtained. The master plates for the present *Prunus material* are shown in figs. 1 and 2.

Estimation of the surfaces of the spots was made by comparing them with a series of ellipses with known surfaces, drawn on a piece of paper.

Statistical procedure. So far, most of the chemotaxonomical studies have dispensed with a closer statistical analysis of the results. It has been the gross, qualitative variation rather that has been made use of in interpreting the results.

In order to make better use of the data available, and in order to avoid subjective mistakes in drawing conclusion, it is however quite obvious that a statistical test is necessary.

The principles of numerical taxonomy have been presented in detail in the valuable book of Sokal and Sneath (1963), which shall be consulted among other things for the estimation of the errors of the various similarity measures to be used.

The most convenient similarity measures are the qualitative ones, e.g.

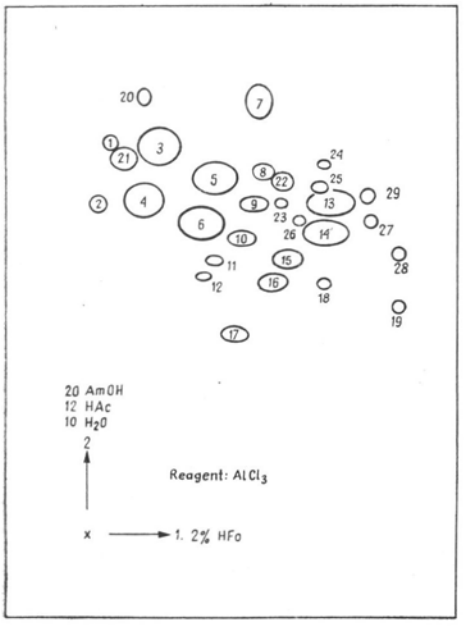


Fig. 1

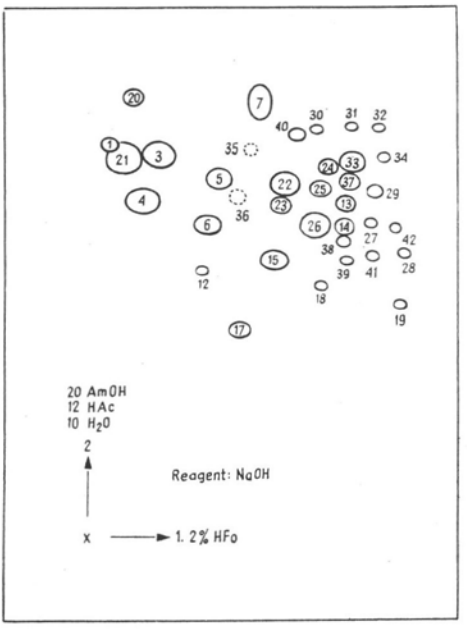


Fig. 2

Fig. 1—2. The chromatographic pattern of leaf phenolics present in analysed *Prunus* species, revealed in UV after spraying with aluminium chloride (1) and sodium hydroxide (2)

either of the following two which in different ways express the proportion of substances that are common to two taxa compared with each other.

$$\text{Matching coefficient, } C_m \frac{p+n}{p+n+d}, \text{ and}$$

$$\text{coefficient of similarity, } C_s = \frac{p+d}{p},$$

where $p$ = number of positive matches, i.e. substances occuring in both taxa; $n$ = number of negative matches, i.e. substances absent in both taxa; $d$ = number of differences.

These coefficients only make use of the qualitative variation between taxa. But a glance at plates from different taxa shows, that it is not just a qualitative variation but more often a quantitative one, the same substances occuring in very different amounts in different plants.

Therefore, the following coefficients, based upon the quantitative variation seem describe better of the material available. They are, however, somewhat more tedious to calculate, especially the correlation coefficient.

$$\text{Correlation coefficient} = \frac{Sxy - \frac{Sx \cdot Sy}{n}}{\left(Sx^2 - \frac{(Sx)^2}{n}\right)\left(Sy^2 - \frac{(Sy)^2}{n}\right)}$$

and biochemical distance $= S(A_{ij} - A_{ik})^2$

For the *Prunus* material analysed, all the four similarity coefficients have been calculated (cf. Table 2).

Tabela 2

Different similarity measures for comparisons of two *Prunus* species and their putative hybrid

| | *P. avium* :*P. fontanesiana* | *P. mahaleb* :*P. fontanesiana* | *P. avium* :*P. mahaleb* |
|---|---|---|---|
| Coefficient of similarity | 0.64 | 0.38 | 0.23 |
| Matching coefficient | 0.64 | 0.50 | 0.29 |
| Correlation coefficient | 0.22 | 0.67 | —0.46 |
| Biochemical distance | 20.175 | 19.175 | 41.500 |

## RESULTS AND DISCUSSION

In figures 10, 11 and 12 UV contact copies of plates from the three taxa involved are presented. The dark spots are the UV-absorbing substances separated. The most obvious differences between *Prunus avium* and *Prunus fontanesiana* consist in the addition of certain spots typical for *Prunus mahaleb*, e.g. the large spots corresponding to spots 13 and 14 in fig. 8. Another such difference seems to be the spot 8, typical of *mahaleb* and occuring in *fontanesiana*.

The original plates with their fluorescent spots in various typical colours still more strong supported the view that *Prunus fontanesiana* is indeed a true hybrid between *Prunus* avium and *Prunus mahaleb*.

Statistical treatment of the data confirms this conclusion. The coefficient of similarity reaches its highest value when comparing *P. avium* with *P. fontanesiana*. The result is in good agreement with conventional taxonomy because *P. fontanesiana* is morphologically much alike *P. avium*. The coefficient has a lower value when comparing *P. mahaleb* with the hybrid, but still lower when comparing both parental forms. The matching coefficient shows very clearly that the hybrid is much more similar to both the parental forms than the parental forms to each other.

The situation is the same as regards the correlation coefficient; it reaches its lowest value when comparing both parental forms with each other. The biochemical distance also shows the great affinity of the two parental forms to the hybrid, in contrast to both parental forms when

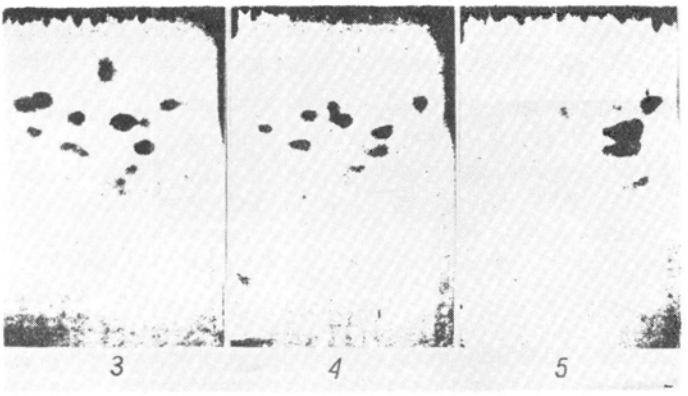


Fig. 3—5. Contact copies made in UV light, showing UV-absorbing phenolic compounds of *Prunus avium* (3), *Prunus fontanesiana* (4) and *Prunus mahaleb* (5).

compared to each other. In contrast to the other coefficients, the biochemical distance places the reputed hybrid at the same distance between both "parents". Very much the same situation is found with regard to the biochemical relationships between the "synthetic" *Prunus cerasus* and its true parents, *Prunus avium* and *Prunus fruticosa* (Oldén and Nybom 1968). Even in this case *P. avium* has a larger number of phenolic substances in its leaves, *P. fruticosa* contributing a smaller number. It seems that in such cases the biochemical distance may give a somewhat better measure of the true relationships. The matching coefficient then also seems to be slightly better than the coefficient of similarity.

The fact that *P. fontanesiana* is supposed to be a hybrid between *P. mahaleb* and *P. avium*, was mentioned by Rehder (1949, cf. also Bailey 1947) although the hybrid was morphologically more alike *P. avium*.

The first effort to utilize the biochemical properties of *Prunus* to identify different species of the genus, was made as early as 1932 (Rawlins and Jarvis 1932). They found, that pieces of bark of *Prunus avium*, boiled in water for 2—5 min., developed yellow-orange colour, while those of *P. mahaleb* did not.

More recently, Bate-Smith (1961) submitted the genus *Prunus* to an extensive biochemical study. He found that all sections of the subgenus *Cerasus* were characterized by a special cherry factor, and what is more that both *P. mahaleb* and *P. fontanesiana* contain a certain *"mahaleb"* factor, not present in *P. avium*. The latter component, which was identified as hernianin, would correspond to the spots No. 13 and 14 in our Table 21 and fig. 8, whereas the cherry factor probably is found in spot No. 33.

Our spots No. 3 and 5, occurring in all the investigated types no doubt are made up of kaempferol glycosides, glucoside and rutinoside, respectively, parallelly to the quercetin glycosides in spots No. 4 and 6.

Spots No. 30—32 are probably hydroxycinnamic acids, whereas the green spots 21—28 seem to be various chlorogenic acid isomers. Spot No. 21 might be iso-chlorogenic acid.

## SUMMARY

With the aid of a special system of thin-layer chromatography, leaves of the three *Prunus* species, *P. fontanesiana*, *P. mahaleb* and *P. avium* were submitted to a chemo-taxonomical investigations. *Prunus fontanesiana* has long been supposed to be an old hybrid between the other two species. Most of the phenolic compounds found in the *Prunus fontanesiana* leaves were present also in *Prunus avium*. Some of them were, however, absent in *Prunus avium* but could be found in *Prunus mahaleb*.

This must be taken as a rather strong indication that *Prunus fontanesiana* is actually a hybrid between *P. avium* and *P. mahaleb*. This concept was further confirmed by statistical treatment of the results.

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## *Badania zależności między Prunus avium, Prunus mahaleb i Prunus fontanesiana przeprowadzone przy pomocy chromatografii cienkowarstwowej*

Streszczenie

W artykule opisano uproszczoną wersję chromatografii cienkowarstwowej, zastosowaną do celów systematyki biochemicznej. Zbadano zależność między *Prunus fontanesiana* oraz przypuszczalnymi formami rodzicielskimi tego mieszańca, tj. *Prunus mahaleb* oraz *Prunus avium*. Stwierdzono, że *Prunus fontanesiana* jest rzeczywistym mieszańcem między *Prunus avium* i *Prunus mahaleb*. W badaniach zastosowano statystyczne mierniki podobieństwa (współczynnik korelacji, dystans biochemiczny, współczynnik podobieństwa oraz współczynnik zgodności).