June, 1984
Kor. Jour. Pl. Tax.
Vol. 14, No. 1, 59–69

Serotaxonomy: a promising approach to systematic problems

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Before I get into some extents, let me have some time to talk on the general remarks on chemical and/or serological characters being employed for classification, phylogeny and evolution.

With the development of plant natural product chemistry, we know well that it is possible to employ chemical constituents to help to characterize, classify, and describe taxa. Interest in this type of research has increased, as more data have been obtained from biochemical, immunochemical and organic chemical research. The development of relatively quick and simple analytical techniques has hastened the "coming of age of chemotaxonomy."

There are two large classes of compounds with potential taxonomic usefullness. The one is the high molecular weighted and essentially polymeric molecules of primary constituents such as nucleic acids and proteins, and the other is relatively low molecular weighted and non-polymeric metabolic end-products such as free amino acids, alkaloids, glycosides, terpenoids etc. commonly known as secondary consituents (Takhtajan 1973).

In spite of the wealth of chemical information, only a few systematically meaningful interpretation have emerged. Because even if we know the biosynthetic origin of all the existing secondary compounds, it would be more difficult to detect evolutionary convergence than the biosynthetic origin. Therefore, the micromolecular approach to taxonomy seems to be of limited value at the moment.

The amount of evolutionary and taxonomic information increases from secondary constituents to proteins, and from proteins to messenger RNA molecules and to genes. Along with the rapidly increasing protein approach to taxonomy, we have witnessed also an equally rapid increase of the use of the nucleic acids. The determination of the proportion of 4 bases in DNA, molecular hybridization, nucleotide sequence study etc. are being attempted although there is no sufficient technique yet.

Today's topic, I want to discuss with you, is concerned to protein approach using serological techniques to systematics and evolution. As we know, the genetic information is directly reflected in proteins via transcription and translation, in other words, protein may be thought of as functional translations of operational unit of DNA. Protein may be considered as a vital link in the chain of events resulting in revolutionary change in terms of systematics.

Now let me get into serology. Serological comparison of proteins is aimed at comparing the total protein stock of taxa. Its use in taxonomy is based on the idea that: (1) each kind of living organism has its own characteristic proteins; (2) the proteins of closely related organisms are closely similar; and (3) those of organisms more distantly related are less alike.

Serological techniques detect similiarities in amino acid sequence and configuration of determinant groups on protein molecule, and thereby provide a measure of structural correspondence among proteins from different taxa. These protein-similarities and differences, when combined with other characters, can help to express evolutionary relationships.

The basic methods for serosystematic or serotaxonomic research involve the immunization of experimental animals to induce antibodies followed by the analysis of reaction of the antibodies produced with properly prepared antigenic material. Reference reaction is referred as the reaction between an antibody and the particular kind of antigenic material used in its formation. Cross reaction is the reaction between an antibody and any antigenic material other than the antigenic material used in its formation.

Systematists and taxonomists have learned that the comparison of antigenic determinants from various taxa help in determining protein similarities which aid our understanding of relationships. It is the specific reactions between antigenic determinants and antideterminants which are valuable because they provide a means for the measurement of protein similarities. Various types of precipitin reactions have been used in plant comparative serological investigations, particularly immunoelectrophoresis had much attention by many researchers. This is based on two of their properties: ability to precipitate in agarose gel with specific antigen and antibody respectively, and electrophoretic mobility in an electric field.

Last decade was a rich period of methodological development, having now immunoelectrophoretic techniques extremely useful for all protein researchers. Some outstanding modification and application of immunoelectrophoresis have appeared recently, but very few systematists have attempted to apply to plant taxa for systematic purpose.

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Much of the serotaxonomic research that has employed immunoelectrophoresis has used the technique of one-dimensional immunoelectrophoresis (Grabar 1959). The disadvantage of this technique lies in the inability to directly compare immunoprecipitin systems of in the analysis of Ouchterlony double diffusion.

A modification of one-dimensional immunoelectrophoresis is crossed immunoelectrophoresis. This technique, described by Axelsen et al. (1975), couples one-dimensional immunoelectrophoresis with a second electrophoresis into a gel containing antiserum (Fig. 1). This double electrophoresis produces a myriad of immunoprecipitin bands (or arcs) when incubated for appropriate time. The distance migrated in the second dimension depended on (1) the initial concentration of each antigen and (2) stability of the precipitin complex that formed when the concentration of each migrating antigen reached equivalence with the immobilized antibodies. Using crossed immunoelectrophoresis, precipitation is performed in an antibody-agarose and takes the form of visible immunoelectrophoretic bands. The immunoprecipitin produced by the protein extracts (antigen systems) to a particular antiserum

can then be rated as to the number of immunoprecipitin bands and the degree or correspondence of bands of the cross-reacting extracts with the reference reaction (Table 1-4; Lee 1981 a,b; Petersen 1983).

Additional technique of radial immunodiffusion is useful for comparision of data obtained from crossed immunoelectrophoresis (Table 1-4; Gleich et al. 1974; Lee 1979; Lee 1981a).

Lee (1981a) using pollen proteins extracted from members of the Asteraceae were among the first experiments to include members of this family. Pollen proteins from seven species of *Ambrosia* and one species from each of five other genera of Asteraceae were compared using the following serological techniques: Ouchterlony double diffusion, radial immunodiffusion, and crossed immunoelectrophoresis. The obtained data supported other taxonomic data which suggested the merging of the genus *Franseria* with *Ambrosia* (Lee 1981a).

Table 1-4. Number of radial immunodiffusion rings (RID) (with diameters in mm) and 2-dimensional immunoelectrophoresis (2-D IE) bands formed with reference reactions of *Ambrosia artemissiifolia* (1), *A (Franseria). acanthicarpa* (2), *Helianthus annus* (3), and *A. trifida* (4) after antisera (As) were presaturated with pollen protein (Ag) from various species. (Ag E) antigen E; (As-Ag) antiserum presaturated with antigen mixture, (aa) *A. artemisiifolia;* (ab) *A bidentata;* (ap) *A. psilostachya;* (at) *A. trifida;* (fc) *A. acanthicarpa;* (fm) *A. ambrosioides;* (ft) *A. tenuifolia;* (ha) *Helianthus annuus:* (hm) *Hymenoclea monogyra.*

1 Ag	RID rings		2-D IE bands	
As-Ag	Ag E	aa	Ag E	aa
aa-0 (non-presaturated)	2-3	7	2	7-8
aa-aa	0	0	0	0
aa-at	0	4	0	4
aa-ab	0	2	0	2
aa-ap	0	2	0	2
aa-fc	0	5	0	1
ff-fm	0	2	0	1
aa-ft	0	4	0	2

2 Ag	RID rings		2-D IE bands	
As-ag	Ag E	fc	Ag E	fc
fc-0	1	7	1	11
fc-fc	0	0	0	0
fc-fm	0	2	0	2
fc-aa	0	4	0	2
fc-ab	0	4	0	3
fc-ap	0	4	0	3
fc-ha	1	5	0	4
fc-hm	1	7	0	6

3 Ag	RID rings		2-D IE bands	
As-Ag	Ag E	ha	Ag E	ha
ha-0	1	7	0	12
ha-ha	0	0	0	0
ha-ha	0	5	0	5
ha-at	0	5	1	6
ha-ab	0	4	0	4
ha-ap	0	3	0	5
ha-fc	0	4	1	3
ha-ft	0	3	1	4
ha-hm	0	4	1	5

4 Ag	RID rings		2-D IE bands	
As-Ag	Ag E	at	Ag E	at
at-0	1	6	1	7
at-at	0	0	0	0
at-aa	0	3	0	3
at-ab	0	3	0	3
at-ap	1	3	0	3
at-fc	1	3	0	3
at-fm	1	3	1	1
at-ft	1	4	1	4
at-ha	1	4	0	4
at-hm	1	6	0	7

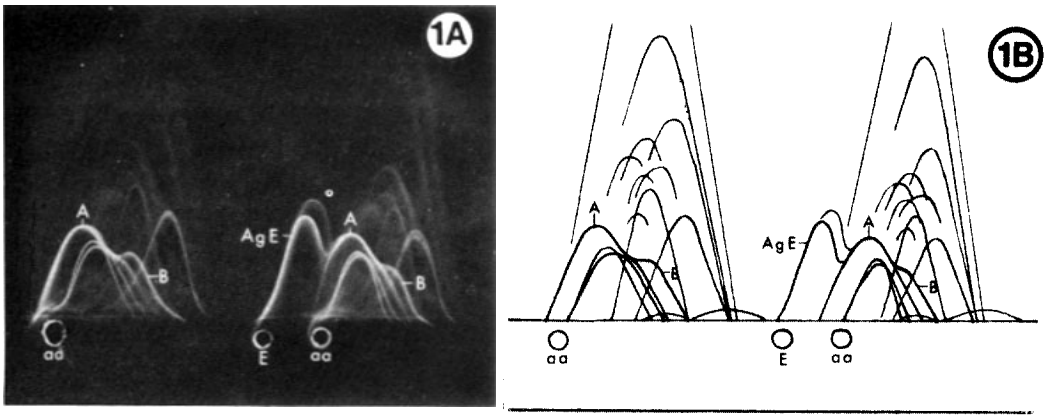

Fig. 1. Crossed immunoelectrophoresis plate of a whole pollen extract of *A. artemisiifolia* alone (left) and in tandem with purified AgE from *A. artemisiifolia* (right). IA, photograph; IB, sketch of precipitin bands. The first dimension consisted of electrophresis (2 hr at 2 V/cm) in buffered agarose and the anode was to the right of the three wells in which samples were placed initially. The second dimension consisted of electrophoresis (15 hr at 1.5 V/cm) into agarose which contained rabbit antiserum against whole pollen extract of *A. artemisiifolia,* and the anode was at the top. The wells labeled "aa" received concentrated pollen extract which represented 2.6 mg of pollen. The well that is labeled "E" received 7 g of AgE. The photograph was taken 58 hr after termination of electrophoresis. (After Lee and Dickinson 1979).

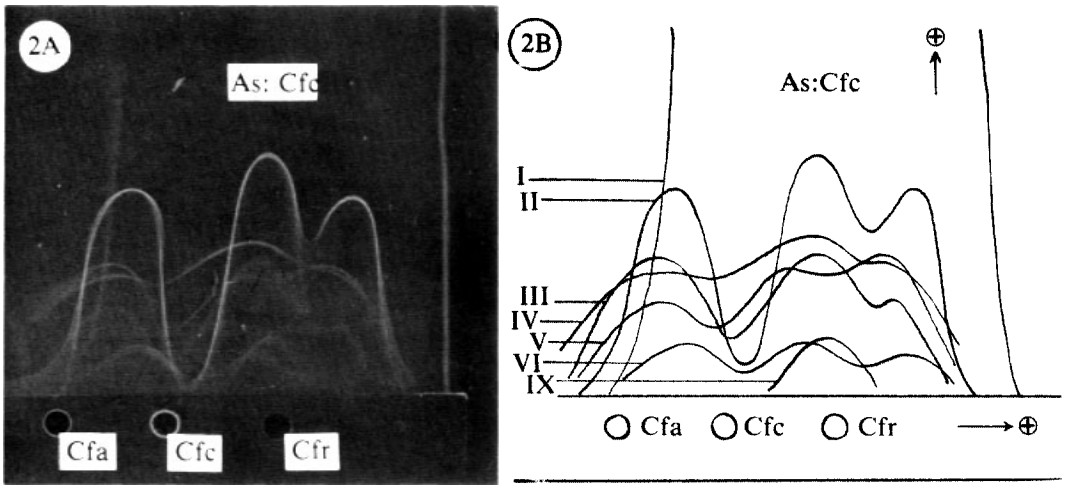

Fig. 2. Tandem-crossed immunoelectrophoresis comparison of three species of *Coffea* with *C. canephora* antiserum. 2A, photograph; 2B, sketch of precipitin bands. Note the unique band IX in *C. canephora.* Antigens: (Cfa) *C. arabica;* (Cfc) *C. canephora;* (Cfr) *C. robusta.* (After Lee 1977)

A modification of crossed immunoelectrophoresis is tandem-crossed immunoelectrophoresis. In this technique, two or three wells are punched in the same track in the first dimensional electrophoresis gel with an intercenter distance of 1cm. (Fig. 2). The samples are applied and allowed to become completely absorbed in gel. Crossed immunoelectrophoresis combined with this procedure also allows one to compare qualitatively and quantitatively two or three complex antigen systems. By producing fusion of related immunoprecipitin systems, starting from two or three adjacent sample wells, one can compare an antigen system with others (Lee 1981b).

Figure 2 shows the tandem-crossed immunoelectrophoresis of three antigenic samples. The wells were placed sufficiently close to each other that identical precipitin peaks from the respective samples were able to fuse, producing triple peaks in the final patterns. Bands I to VI were present in all three species. These antigenic materials were identical in all species because each band forms a continuous line. *Coffea robusta* was generally lower in antigenic materials. Because of variation in the shape of the peak, their heights were only approximations of the amounts of antigenic materials present. Band IX in *C. canephora* obviously had no counterpart in extracts of the other species. The advantage of tandem-crossed immunoelectrophoresis as shown in this experiment (Fig. 2) was that many heterogenous proteins from the three species can be compared semiquantitatively upon the same plate. However, its disadvantage is that the continuous line, which resulted from each identical band from the three species, is not clearly connected in many cases. Sometimes the connected portion of identical bands originating from the different wells in tandem is too faint to read and/or overlapped nonidenical bands, giving misleading results. It is to be noted that band IX is isolated but not connected to band VI of *C. arabica* and *C. robusts*. Therefore, the height of each peak should be confirmed by individual crossed immunoelectrophoresis to overcome this disavantage.

Very recent contribution of serological protein properties to phylogeny and taxonomy includes researchs of Lester et al. (1983), Fairbrothers and Petersen (1983), Smith (1983), Cristofolini and Peri (1983), Klozova et al. (1983), Prus-Glowacki (1983), and Dahlgren (1983).

In phylogenetic research the protein characters to be compared must be easily analyzed by methods suited to the construction of cladistic schemes on *Solanum*. Such methods has been proposed for serological data (Lester et al. 1983).

Serological data obtained from seed meal extracts 16 taxa from 5 families including Magnoliaceae were analyzed by means absorption similarity coefficients by Fairbrothers and Petersen (1984). Using unweighted pair group method analysis (UPGMA), dendrograms of serological similarity were generated.

Prus-Glowacki (1983) using serological techniques proved that the antigenic properties of individuals from a hybrid swarm population of *Pinus sylvestris* x *P. mugo* allowed the establishments of characteristics, and the degree and direction of introgression in the hybrid population.

Dahlgren(1983) had demonstrated the importance of serological date for helping to formulate conclusions phylogenetic relationships in connection with their role in the taxonomy

of higher levels. He also emphasizes the importance of understanding the methods and materials used in serotaxonomic investigations before coming to valid conclusions. He clearly demonstrates that serological data support some views and contradicts others, as well as open new alternative taxonomic interpretations.

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Now I am going to get into a practical application by introducing my current serotaxonomic investigation of the genus *Quercus* in Korea.

A review of the taxonomic treatment of *Quercus* taxa native to eastern Asia reveals several different treatments. Nakai (1952) or Chung (1957) placed the taxa in 2 genera (*Cyclobalanopsis* and *Quercus*). Lee (1961) or Makino (1961) had one genus with 2 subgenera (Cyclobalanopsis and Lepidobalanus). According to Western taxonomists, Oersted (1871) or Tucker (1979) placed these taxa in 3 subgenera (Erythroblalanus, Leidobalanus, and Macrobalanus) under the genus *Quercus*. In Schwarz's treatment (1936), Erythrobalanus and Macrobalanus were elevated to generic rank and Lepidobalanus and another subgenus Scherophyllodrys were retained as subgernera of *Quercus*. Melchior (1964) designated Cyclobalanopsis, Erythrobalanus, and Lepidobalanus within the *Quercus*. Thus, the intra- and intergeneric taxonomic problems, and proper relationships remain to be resolved.

Therefore, it has become necessary to investigate new characteristics and attempt to determine the significance of these characteristics in terms of *Quercus* taxonomic treatments. To obtain additional data as an aid in the interpretation of relationships, pollen proteins were employed in this research.

Preparation of immunoplate for rocket immunoelectrophoresis was designed in order to maximize advantages of this technique, and not to waste antiserum. The heated agarose solution was poured directly on the antibody-containing gel. And each protein sample was placed in each well. The electrophoresis was performed at 2 V/cm for two hours at 8°C. The observed rocket are due to the gradient of polyvalent antiserum, and well defined (Lee 1983).

In Fig. 3, the upper portion represents the photograph of rocket patterns at a particular moment, whereas the lower portion shows all the immunoprecipitin system appeared during incubation time. Although some of them were too faint to sketch and did not appear on the photograph, all of them were included to count. In these preliminary experiments quantitation of all the number of rocket and all available rocket heights were condidered. It was difficult to identify each corresponding rocket among taxa with this technique. However, it is worthy to note that two major immunoprecipitin systems showing the sharpness with high intensity in *Q. dentata* reference reaction were shared in many species. One of these 2 systems remained near the well in which the sample was applied, and the other was relatively highly charged and moved rapidly toward anode. The former and latter are tentatively designated as major immunoprecipitin systems m_1 and m_2. With *Q. dentata* antiserum, extracts from *Q. aliena, Q. dentata, Q. donarium, Q. sersata, Q. acutissima,* and *Q. acutissima* x *variabilis* revealed m_1 with their rocket heights from 4 to 6.5mm. And extracts from these species except *Q. acutissima* and *Q. acutissima* x *variablis* showed m_2 with their rocket

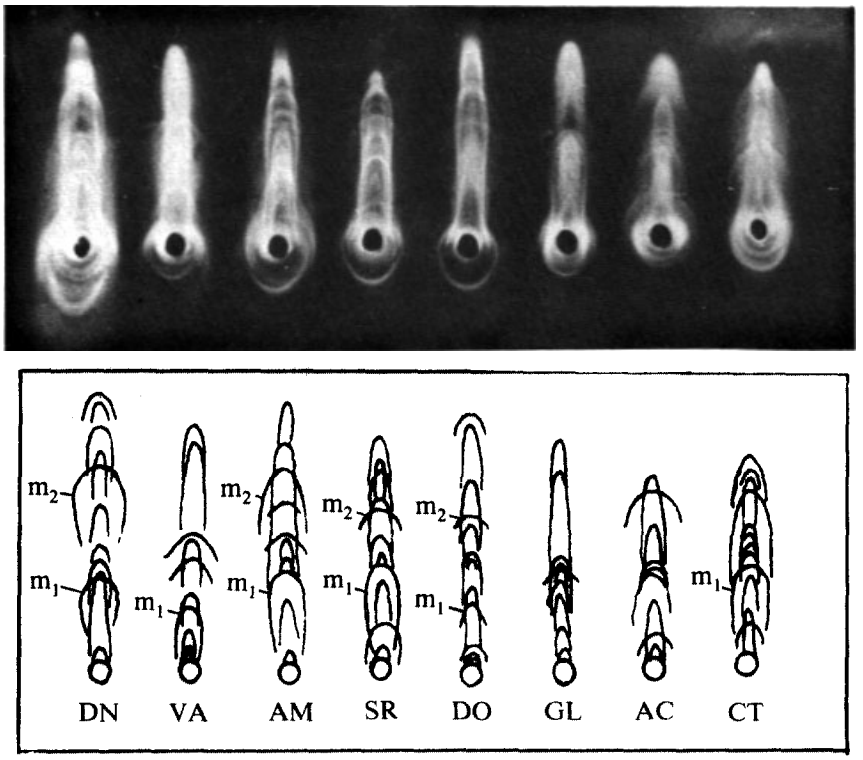

Fig. 3. Above: Rocket immunoelectrophoretic patterns. The agarose gel was charged with *Quercus aliena* antiserum. Below: Sketches of the rockets from photograph. This technique was used for quantitative determination of a protein in a protein mixture, and was used to further characterize the *Quercus* antigenic materials. Agarose gels containing monovalent antiserum were used in previous works with rocket immunoelectrophoresis but poyvalent antiserum was used in serotaxonomic research. It was difficult to identify each band with this technique, but these can be verified by other methods (e. g., 2-dimensional immunoelectrophoresis). When the antigenic materials from the species of *Querucs* were electrophoresed on the same plate, the from and shape, the distinctness, and the ability to take up staining in bands all provide additional qualitative information for interpretation. In Figure 3 the most predominant bands in most species were m_1 and m_2. Antigens: (DN) *Q. dentata;* (VA) *A. acutissima* x *variabilis;* (AM) *A. aliena:* (SR) *Q. serrata:* (DO) *Q. donarium;* (GL) *Q. glauca;* (AC) *A. acuta;* (CT) *Q. acutissima.* (After Lee and Cho 1981)

heights from 9.5 to 13mm. Extracts from *Q. acuta* and *Q. glauca* revealed neither m_1 nor m_2 in the same agarose plate. These data indicate that *Q. dentate* is serologically close to *Q. aliena, Q. donarium,* and *Q. serrata;* less close to *Q. acutissima* and *Q. acutissima* x *variabilis;* and least close to *Q. acuta* and *Q. glauca.*

As revealed in Table 5 rocket height data were used as an index of protein similarity or serological correspondence (SC). Serological correspondence is expressed as a percentage

Table 5. Quantification of rocket heights (mm) for eight species of *Quercus* obtained by rocket immunoelectrophoresis. The agarose gel was charged with *Q. aliena* antiserum. Secrological correspondence (SC) is the percentage value of total rocket heights in the reference reaction (cross reaction/reference reaction x 100). Gothic numbers are the rocket heights of the two detected major immunoprecipitin systems (After Lee 1983)

Taxa	Rocket Heights	Number of Rockets	Total Rocket Heights	SC (%)
Q. acuta	1, 2, 2.5, 3.5, 5, 6, 7, 8, 11, 15	10	61.0	66
Q. acutissima	3, 3.5, 5, **6**, 6.5, 7, 8, 9.5, 12, 13.5, 14.5, 16	12	104.5	112
Q. acutissima x *variabilis*	0.5, 1.5, **4**, 5, 7, 9.5, 10, 15, 16.5	10	72.0	77
Q. aliena	1, 1.5, 3, **5**, 7, 9, 10, 11, **13**, 15, 18	11	93.5	100
Q. dentata	1, 5, 5.5, 6.5, 8, 10.5, **12.5**, 14.5, 18, 19	11	115.0	118
Q. donarium	0.5, 1, 1.5, 4, **5.5**, 8.5, 9, **9.5**, 13, 17, 19	11	88.5	9.5
Q. glauca	3, 4, 6, 7, 8, 9, 14, 17	8	68.0	73
Q. serrata	1.5, 3, 4.5, **6.5**, 7, 8.5, 9.5, 11, **12.5**, 14, 15, 16.5	11	109.5	115

value of total rocket heights in the reference reaction (cross reaction/reference reaction x 100). The sum of all rockets resulting from a reaction between an antiserum and antigenic material used to stimulate its formation (reference reaction) was designated as 100%. Serological comparisons among taxa obtained by this procedure are presented in Table 1. Antisera produced to *Q. aliena* and *Q. donarium* produced strong reactions resulting in high rockets with *Q. aliena, Q. dentata, Q. donarium, Q. serrata, Q. acutissima,* and produced relatively weak reactions with *Q. acuta, Q. glauca* and *Q. acutissima* x *variabilis.* Theoretically the maximum quantification of immunoprecipitin systems, that is 100% in serological correspondence, would be expected in the reference reaction. The values slightly over 100% in the cross reaction in Table 1 reflect experimental error. Therefore, serological correspondence should be used as a relative value for comparisons, but not as absolute ones.

When conventional immunoelectrophoresis was applied to antigenic materials from various *Quercus* species and the trough was charged with *Q. aliena* antiserum, the results were as follows. *Quercus aliena, Q. dentata,* and *Q. donarium* belonging to Lepidobalanus have 2 major immunoprecipition systems, m_1 and m_2. *Q. acutissima* and *Q. acutissima* x *variabilis* in Lepidobalanus have 1 major immunoprecipitin system, m_1 only. But *Q. glauca* of Cyclobalanopsis has m_1 with slightly different electrophoretic mobility, and *Fagus glandifolia* has 1 immunoprecipitin system similar to m_1 and not major leading (Lee 1981 b).

Overall quantitative and qualitative serological data indicate that the subgenus Cyclobalanopsis is distinct from Lepidobalanus. However, the elevation of the Cyclobalanopsis to the rank of a separate genus does not seem warranted. An understanding of the relationships of Lepidobalanus to Erythrobalanus requires additional research.

Figure 4 shows an imaginary phylogenetic tree of the genus *Quercus* and *Fagus,* which is

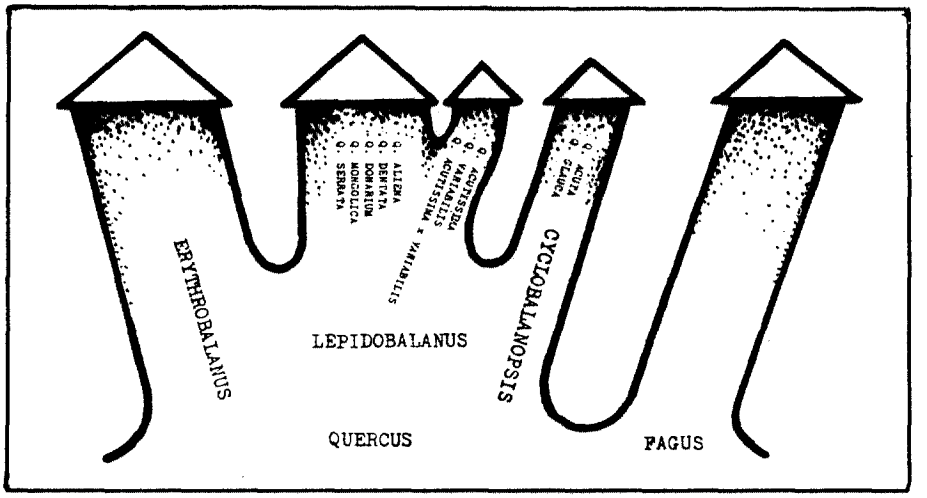

Fig. 4. Possible phylogenetic relationships among serological groupings of *Quercus* and *Fagus*.

based a overall serological data obtained. *Quercus* and *Fagus* are the separate genera. Within *Quercus,* there are 3 distinct subgenera: Erythrobalanus, Lepidobalanus, and Cyclobalanopsis. Within subgenus Leipidobalanus, there are 2 groupings which are considered to be on the way of separating into genera sooner or later. *Quercus acutissima-Q. variabilis* line tends to be separating from the major stock of Lepidobalanus.

In this preliminary research, rocket immunoelectrophoresis proved to be valuable for use in serotaxonomy. To obtain a protein similarity or serological correspondence the sum of total rocket heights proved useful. Rocket patterns made it possible to measure individual immunoprecipitin systems which were identical or partially identical to those systems detected in reference reactions and quantification of all available rocket heights such serotaxonomic approach is one of multidesciplinary approaches for systematic purpose. It is becoming more and more obvious that no single method can provide fully confident information on evolutionary classification. No single source of information can substitute for integrative approach to plant classification based on the correlation and synthesis of the evidence taken from all available source of knowledge. Therefore, serological data plus other discplinary information can introduce any fundamental changes into the present day classification system.

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