The free amino acids of pollen of some angiospermic taxa as taxonomic markers for phylogenetic interrelationships

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Cluster pairing affinity or similarity index between eight interrelated families of angiosperms, namely Acanthaceae, Bignoniaceae, Boraginaceae, Convolvulaceae, Labiatae, Scrophulariaceae, Solanaceae and Verbenaceae was evaluated on the basis of the free amino acid composition of pollen as taxonomic markers of five members of each family, to assess the phylogenetic interrelationships and plant affinity. Considerable amount of homology was observed in the species belonging to the same family. Homology being more pronounced within species belonging to the same genus. Considerable homology was also observed between the various families. Amino-*n*-butyric acid, aspartic acid, glutamic acid, methionine, phenylalanine and proline were the major amino acids found in the various pollen. Dendrogram constructed on the basis of free amino acid composition places the eight families in two clusters, with Verbenaceae and Labiatae being the most closely related in one group, and Scrophulariaceae and Bignoniaceae in the other. Scrophulariaceae also shows similarity with Acanthaceae and Solanaceae, with the latter being closely related to Convolvulaceae. Boraginaceae, showing a high degree of similarity with Verbenaceae and Labiatae, was also found to be related to Convolvulaceae. The present observation will open a new vista using free amino acids of pollen as taxonomic markers in the conservation of biodiversity.

Keywords: Free amino acids, pairing affinity, phylogenetic interrelationship, pollen.

POLLEN, the male partner in the fertilization process of flowering plants, because of its unique structure and haploid nature, provides an excellent material for several physiological and biochemical studies of fundamental and applied nature, which can give interesting clues regarding storage, longevity, viability, different phases of pollen development, biogenesis of wall, polysaccharides of pollen tubes, etc., thus enabling scientists to elucidate certain basic patterns and mechanisms of plant growth, giving an improved understanding of many basic cell processes and insights to improve the yields of desired crops. Further, pollen is a major source of morbidity for atopic patients, leading to various allergic disorders[1–7]. A study of pollen biochemistry can provide basic data to aeropalynologists and allergologists, which can help them understand the role played by the various chemical constituents of pollen in the allergic manifestations. However, due to various technical difficulties in collecting uncontaminated pollen, critical knowledge regarding pollen biology and biochemistry still remains fragmentary and mostly remains confined to taxa having large anthers and particularly anemophilous plants which produce a large amount of pollen, leading to a serious limitation in the understanding of the physiology and biochemistry of pollen development[8]. Thus, in recent years scientists are showing special interest in pollen physiology and biochemistry, particularly the role of various enzymes and other biochemical constituents in different structural and functional aspects.

Pollen has also proved to be an excellent tool in taxonomic studies. The application of pollen characters in solving controversial taxonomical and phylogenetic problems, has now been widely recognized all over the world[9]. Application of pollen characters and constituents in understanding plant affinity and phylogeny is also well documented[10–13]. However, these studies have been largely confined to major morphological characters of pollen grains, including apertural form, number, distribution and position[14], exine ornamentation and stratification patterns, pollen association and pollen nuclear number, all of which have provided the best taxonomic criteria, being least variable[15,16]. A central problem of phylogenetics has been to distinguish between the component of biological similarity due to descent from common ancestry (homology), and that due to convergence from different ancestors (analogy), i.e. evolutionary classification should reflect only true homologies. But morphological characters and behavioural patterns which have till recently provided the major guidelines to classification, can sometimes evolve independently in unrelated species in response to

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Table 1. Total free amino acid content (%) of the pollen of 40 angiospermic plants belonging to eight families (data based on ten readings for each species)

Plant	Mean value of total free amino acid content (μmol/mg dry wt)	sd
Acanthaceae		
Adhatoda zeylanica Medic. Hist. & Commetat.	5.12	0.089
Andrographis paniculata Nees.	7.73	0.098
Barleria cristata L.	3.40	0.076
Justicia simplex Don.	7.84	0.092
Ruellia tuberosa L.	1.60	0.063
Bignoniaceae		
Jacaranda acutifolia L.	5.35	0.086
Kigelia pinnata DC.	1.90	0.068
Pyrostegia venusta (Ker-Gawl.) Miers.	6.00	0.090
Spathoda campanulata Beaw	0.58	0.048
Tecoma stans (Linn.) H.B. & K. Nov.	2.48	0.064
Boraginaceae		
Cordia dichotoma Forst.f.	1.30	0.054
Heliotropium arborescens L.	1.36	0.058
H. indicum L.	1.16	0.050
H. ovalifolium Forsk.	2.10	0.061
Trichodesma indicum R.Br	1.41	0.056
Convolvulaceae		
Argyreia nervosa Boj.	2.06	0.060
Convolvulus arvensis L.	5.62	0.089
Cuscuta reflexa Roxb.	5.62	0.086
Ipomoea fistulosa L.	2.90	0.066
I. pes-tigridis L.	1.96	0.063
Labiatae		
Anisomeles indica O. Kuntze	4.30	0.074
Hyptis suaveolens Poir.	5.40	0.083
Leucas aspera Spreng.	4.51	0.076
Ocimum sanctum L.	3.55	0.063
Salvia splendens Ker-Gawl.	4.45	0.070
Scrophulariaceae		
Antirrhinum majus L.	2.65	0.063
Bacopa monnieri Pennel	1.90	0.068
Digitalis purpurea L.	3.50	0.071
Lindenbergia indica (L.) O. Kuntze	4.85	0.081
Scoparia dulcis L.	3.85	0.077
Solanaceae		
Datura metel L.	1.81	0.063
Physalis minima L.	3.70	0.070
Solanum indicum L.	4.12	0.074
S. nigrum L.	6.85	0.091
S. surattense Burm. f.	1.55	0.056
Verbenaceae		
Clerodendrum phlomoidis L.f.	7.82	0.093
C. petasites (Lour.) Moore	6.50	0.084
C. splendens G. Don.	5.20	0.073
Lantana camara L.	5.82	0.074
Vitex negundo L.	2.28	0.030

sd, Standard deviation.

common environmental challenges[17]. According to Erdtman[18], evolution depends upon a combination of internal and external factors such as mutation, recombination of generic differences and selection, and during evolution it may happen that unrelated groups of plants give rise to morphologically similar ones, which he called 'convergence' or 'parallel development'. Conversely, related plants may give rise to very dissimilar descendants (divergence). Such phenomena can cause considerable taxonomic difficulties. Thus chemical taxonomy, which is based on the

investigation of the distribution of chemical compounds in a series of related or supposedly related plants has proved to be important in providing additional knowledge of phylogeny or presumed homology, and in this regard, pollen seems to have been least exploited. There are a few earlier works on the use of chemical constituents of pollen in understanding plant affinity and phylogeny[9,19–23].

This article will focus on the total free amino acid content and free amino acid composition of the pollen of 40 angiospermic species belonging to eight families as taxonomic markers and based on the assessment of homologies in the amino acid composition, we have tried to resolve the relationships among species belonging to the same family and also the phylogenetic interrelationships among the eight related families of Acanthaceae, Bignoniaceae, Boraginaceae, Convolvulaceae, Labiatae, Scrophulariaceae, Solanaceae and Verbenaceae. Pollen for a particular species was collected from several individual plants of the same species to obtain a constant chemical composition, as pollen chromatograms show great variation at different stages of maturity and with different environmental conditions. The variation in amino acids resulting from differences in handling and storage was avoided by harvesting all the pollen and rapidly drying over silica gel at 30°C, following the method of Pfahler and Linskens[24]. Though the amino acid content can vary with the climatic and nutritional conditions of the plants on which the pollen matures, analysis has revealed the presence of all the essential amino acids in pollen[25]. The total level of free amino acids is usually higher in the pollen than in leaves and other tissues. At the same time, the concentration of all amino acids in the pollen is considerably higher in bound form than in the free fraction. Proline is one of the most abundant free amino acids found in pollen. The other free amino acids reported in pollen include α–β-alanine, α-amino-n-butyric acid, arginine, aspartic acid, cysteine, ethanolamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine and valine[25]. Besides these, certain unusual free amino acid-like compounds have also been reported in pollen, for example, pipecolic acid in the pollen of *Rosa domascena*, taurine in five species of bee-collected angiosperm pollen, spermine and spermidine in *Petunia* pollen, etc.[25].

Methodology

Pollen was collected from mature anthers (after anthesis) of all the 40 plant taxa, from plants growing in and around Santiniketan, West Bengal. The flowers were allowed to dehisce under natural conditions and the pollen was shaken free from the anthers by placing them in dry polyethylene containers. The pollen was then collected by sieving through meshes of different size (100, 200 and 300 μm) to remove the anther walls and other debris. To prevent the variation of free amino acids due to handling and storage, the pollen was rapidly dried over silica gel at 30°C immediately after sieving[24], instead of sun-drying and storage over dried silica in a desiccator or freeze-drying. The pollen was then immediately used for biochemical analysis. The pollen materials used in the present investigation belong to 40 angiospermic species of eight different families (Table 1). The pollen were collected separately from at least 20 different individual plants of the same species and qualitative analysis of free amino acids was carried out separately for each sample collected, i.e. 20 data for each species to know the amino acid composition. From these, five representative pollen samples having all the identified amino acids were selected for quantitative estimation of individual amino acids for each separate plant. However, the pooled data of total amino acid content represented in Table 1 are based on the mean data of ten individual plants of each species.

Free amino acids were extracted from the pollen using the methods of Bieleski and Turner[26]. 100 mg of the sample was ground at –20°C and 4 ml of methanol : chloroform : water (12 : 5 : 3 v/v) was added and vortexed for 2 min, and then centrifuged at 900 *g* for 10 min. The pellet was re-extracted with 2 ml of methanol : chloroform : water, vortexed and centrifuged again for 5 min. The procedure was repeated with 2 ml of 80% ethanol. The supernatants were combined and phase separation achieved by adding 2 ml of chloroform and 1.5 ml of deionized water followed by centrifugation at 900 *g* for 10 min. The aqueous extract was dried under vacuum and amino acids resolubilized in 500 μl of 0.01 M HCl. This extract was used both for quantitative and qualitative analysis of free amino acids.

The total free amino acid content of the pollen was quantified using ninhydrin reagent. To 2 ml of amino acid extract, 2 ml of buffered ninhydrin reagent [0.8 g of ninhydrin and 0.12 g of hydrindantin in 30 ml of methyl cellusolve and 10 ml of acetate buffer (pH 5.5)] was added and the mixture heated on a boiling water bath for 15 min. The solution was then cooled to room temperature and 3 ml of 50% ethanol was added. The extinction of the purple colour developed was read at 570 nm after 10 min using a spectrophotometer. Appropriate blanks were set up and the colour equivalence of the amino acids under investigation was compared. A calibrated solution of glycine was used as standard[26].

Qualitative analysis of the free amino acids of the pollen of the investigated taxa was done using thin layer chromatography (TLC). DC-Alufolien Kieselgel 60 aluminium sheets (Merck) were used for performing TLC, according to the method described by Sadasivam and Manickam[26]. The TLC sheets were activated by heating in an oven for 30 min at 100–120°C, and the amino acid extract spotted on them and chromatographed using *n*-butanol : acetic acid : water (80 : 20 : 20 v/v) as eluant and 0.1% ninhydrin in acetone as spraying reagent. The

Table 2. Free amino acid composition of pollen of five members of Acanthaceae

	Adhatoda zeylanica		*Andrographis paniculata*		*Barleria cristata*		*Justicea simplex*		*Ruellia tuberosa*	
Amino acid	A	B	A	B	A	B	A	B	A	B
Alanine	–	–	+	0.208	+	0.036	–	–	–	–
Amino-*n*-butyric acid	+	1.216	+	1.121	+	0.782	+	0.636	+	0.221
Aspartic acid	–	–	+	1.202	+	0.762	+	0.842	+	0.216
Glutamic acid	–	–	–	–	–	–	+	2.101	–	–
Glycine	+	0.512	+	0.631	+	0.098	+	0.102	+	0.213
Histidine	–	–	–	–	+	0.108	–	–	+	0.103
Leucine	–	–	–	–	–	–	–	–	+	0.096
Methionine	–	–	+	0.212	–	–	+	0.135	+	0.054
Ornithine	–	–	–	–	–	–	–	–	+	0.133
Phenylalanine	–	–	+	1.932	+	0.212	–	–	–	–
Proline	+	2.330	+	+	+	1.206	+	3.162	+	0.463
Serine	+	0.738	–	–	–	–	+	0.413	–	–
Threonine	–	–	–	–	–	–	–	–	+	0.063
Tryptophan	–	–	+	+	+	0.021	–	–	–	–
Valine	+	0.168	+	+	+	0.036	+	0.063	+	0.041
Unidentified	–	–	1	0.092	2	0.020	1	0.368	–	–

A, Free amino acid present; B, Amount in μmol/mg dry wt (data based on mean value of five readings for each species) for Tables 2–9.

Table 3. Free amino acid composition of pollen of five members of Bignoniaceae

	Jacaranda acutifolia		*Kigelia pinnata*		*Pyrostegia venusta*		*Spathodea campanulata*		*Tecoma stans*	
Amino acid	A	B	A	B	A	B	A	B	A	AB
Amino-*n*-butyric acid	+	1.203	+	0.216	+	0.131	+	0.316	+	0.310
Arginine	+	–	+	0.133	–	–	+	0.221	+	0.241
Aspartic acid	+	1.311	–	–	+	0.122	+	1.413	+	0.290
Glutamic acid	+	0.129	+	0.138	+	0.068	–	–	+	0.252
Histidine	–	–	–	–	+	0.021	–	–	–	–
Hydroxyproline	–	–	–	–	–	–	+	3.112	–	–
Isoleucine	+	0.131	–	–	–	–	–	–	–	–
Methionine	–	–	+	0.193	–	–	–	–	–	–
Ornithine	+	0.133	–	–	–	–	–	–	–	–
Phenylalanine	–	–	+		–	–	+	0.322	+	0.260
Proline	+	2.116	+	0.561	+	0.213	–	–	+	0.481
Serine	–	–	+	0.313	–	–	–	–	–	–
Threonine	–	–	–	–	–	–	+	0.218	–	–
Tryptophan	–	–	–	–	–	–	+	0.023	–	–
Tyrosine	+	0.133	–	–	–	–	+	0.096	+	0.192
Unidentified	3	0.156	3	0.292	–	–	1	0.021	1	0.280

amino acids were detected by heating the sheets at 110°C for 5 min and the *Rf* values were calculated. The spots were identified by comparing with the *Rf* values of standard amino acids.

To quantify the amount of amino acid in each spot after chromatography, the samples were chromatographed on two sheets under identical conditions. One of the sheets was sprayed with ninhydrin to identify the spots. The positions corresponding to these spots in the other sheets were scraped-off and taken in a test tube to which 5 ml of 80% ethanol was added for elution. The concentration in the residual supernatant after centrifugation was determined by the ninhydrin method, as mentioned earlier.

The method of pairing affinity or similarity index described by Sokal and Sneath[27] and Romero Lopes *et al.*[28] was used to analyse the data of free amino acid composition and determine the pairing affinity between the eight families. The degree of pairing affinity (PA) between two families was calculated according to the following formula:

$$PA = \frac{\text{Amino acids common to families } A \text{ and } B}{\text{Total amino acids in } A \text{ and } B} \times 100.$$

Results and discussion

On a dry weight basis, relative values for extractable free amino acids from the pollen of the investigated plant taxa were considerably low (below 10%) and ranged between

Table 4. Free amino acid composition of pollen of five members of Boraginaceae

	Cordia dichotoma		*Heliotropium arborescens*		*Heliotropium indicum*		*Heliotropium ovalifolium*		*Trichodesma indicum*	
Amino acid	A	B	A	B	A	B	A	B	A	B
Alanine	–	–	+	0.143	–	–	+	0.096	–	–
Amino-*n*-butyric acid	+	0.058	+	0.212	+	0.212	+	0.101	+	0.102
Arginine	–	–	+	0.093	–	–	+	0.068	–	–
Aspartic acid	+	0.046	+	0.311	+	0.108	+	0.364	+	0.111
Cysteine	–	–	–	–	–	–	+	0.061	–	–
Glycine	–	–	–	–	+	0.061	–	–	–	–
Histidine	+	0.024	–	–	–	–	–	–	+	0.054
Lysine	–	–	+	0.022	–	–	+	0.036	–	–
Proline	+	0.546	+	0.421	+	0.588	+	0.766	+	0.648
Tryptophan	–	–	+	0.054	+	0.042	+	0.042	+	0.123
Tyrosine	+	0.043	–	–	–	–	+	0.075	–	–
Valine	+	0.102	+	0.038	–	–	–	–	+	0.079
Unidentified	–	–	–	–	1	0.101	–	–	2	0.213

Table 5. Free amino acid composition of pollen of five members of Convolvulaceae

	Argyreia nervosa		*Convolvulus arvensis*		*Cuscuta reflexa*		*Ipomoea fistulosa*		*Ipomoea pes-tigridis*	
Amino acid	A	B	A	B	A	B	A	B	A	B
Alanine	+	0.096	+	0.076	–	–	+	0.642	+	0.016
Amino-*n*-butyric acid	+	0.048	+	1.428	+	0.106	+	0.058	+	0.411
Arginine	–	–	+	0.064	–	–	+	0.243	+	0.182
Aspartic acid	+	0.054	+	0.098	+	0.112	+	0.546	+	0.102
Cysteine	–	–	–	–	+	0.096	–	–	–	–
Glycine	–	–	–	–	+	0.074	–	–	–	–
Hydroxyproline	–	–	–	–	+	0.083	–	–	–	–
Leucine	+	0.068	+	0.104	–	–	+	0.438	+	0.098
Lysine	–	–	+	1.096	–	–	–	–	–	–
Phenylalanine	+	0.101	+	0.048	+	0.078	+	0.648	+	0.416
Proline	+	1.468	+	2.464	+	4.611	–	–	+	0.501
Tryptophan	–	–	–	–	+	0.142	–	–	–	–
Tyrosine	+	0.112	+	0.039	–	–	+	0.201	+	0.123
Valine	+	0.108	+	0.102	+	0.211	–	–	+	0.098
Unidentified	–	–	1	0.096	2	0.106	–	–	–	–

Table 6. Free amino acid composition of pollen of five members of Labiatae (= Lamiaceae)

	Anisomeles indica		*Hyptis suaveolens*		*Leucas aspera*		*Ocimum sanctum*		*Salvia splendens*	
Amino acid	A	B	A	B	A	B	A	B	A	AB
Alanine	+	0.083	–	–	+	0.046	–	–	+	0.042
Arginine	–	–	+	0.142	+	0.098	+	0.101	+	0.036
Aspartic acid	+	1.211	+	1.046	+	1.060	+	0.183	+	1.021
Cysteine	–	–	+	0.060	–	–	–	–	+	0.014
Glycine	+	0.098	+	0.079	+	0.078	+	0.248	+	0.074
Lysine	+	0.042	–	–	+	0.084	+	0.361	+	0.036
Methionine	+	1.012	+	1.089	+	0.542	+	1.064	+	1.011
Proline	+	1.681	+	2.612	+	1.712	+	1.431	+	2.016
Threonine	–	–	+	0.121	–	–	+	0.087	–	–
Valine	+	0.099	+	0.164	–	–	+	0.064	+	0.079
Unidentified	–	–	1	0.091	2	0.846	–	–	1	0.102

1.16 and 7.84% (Table 1). *Spathodea campanulata*, however, showed an extremely low level of total free amino acid content, constituting just 0.58% of the pollen dry weight. *Justicia simplex*, a prostrate herb belonging to the family Acanthaceae, having just two stamens, showed the highest level of free amino acids (7.84%), followed by *Clerodendrum phlomoides* (7.82%) and *Andrographis paniculata* (7.73%). All the five members of Boragina-

Table 7. Free amino acid composition of pollen of five members of Scrophulariaceae

	Antirrhinum majus		*Bacopa monnieri*		*Digitalis purpurea*		*Lindenbergia indica*		*Scoparia dulcis*	
Amino acid	A	B	A	B	A	B	A	B	A	B
Alanine	–	–	+	0.095	–	–	–	–		–
Amino-*n*-butyric acid	+	0.120	+	0.063	+	1.012	+	1.666	+	1.014
Aspartic acid	+	0.020	+	0.076	+	0.146	+	0.542	+	1.016
Cysteine	–	–	+	0.042	+	0.164	–	–	+	0.142
Glutamic acid	+	0.080	–	–	+	0,129	+	0.106	+	0.161
Histidine	+	0.020	–	–	+	–	–	–	–	–
Hydroxyproline	–	–	+	0.010	+	0.096	–	–	–	–
Isoleucine	+	0.030	+	0.034	+	0.063	+	0.091	+	0.044
Leucine	+	–	–	–	+	0.020	–	–	–	–
Methionine	+	0.054	+	0.077	–	–	+	0.063	+	0.056
Ornithine	+	–	–	–	–	–	+	0.042	–	–
Phenylalanine	+	0.074	+	0.102	–	–	+	0.016	+	0.083
Proline	+	2.080	+	1.202	+	1.648	+	2.102	+	1.216
Serine	+	0.020	–	–	–	–	–	–	–	–
Threonine	–	–	+	0.096	+	0.073	–	–	+	0.014
Tryptophan	+	0.063	–	–	–	–	+	0.122	–	–
Tyrosine	+	0.054	–	–	+	0.123	–	–	+	0.089
Unidentified	–	–	1	0.068	–	–	1	0.098	–	–

Table 8. Free amino acid composition of pollen of five members of Solanaceae

	Datura metel		*Physalis minima*		*Solanum indicum*		*Solanum nigrum*		*Solanum surattense*	
Amino acid	A	B	A	B	A	A	A	B	A	B
Alanine	–	–	+	0.263	–	–	–	–	–	–
Amino-*n*-butyric acid	+	0.146	+	0.098	+	0.868	+	1.220	+	0.168
Arginine	+	0.154	+	0.121	+	0.586	+	1.220	+	0.234
Aspartic acid	+	0.046	+	0.111	–	–	–	–	+	0.022
Cysteine	–	–	+	0.142	–	–	–	–	+	0.206
Glutamic acid	+	0.032	+	0.162	+	0.418	+	0.463	+	0.052
Histidine	–	–	+	1.016	+	0.084	–	–	–	–
Isoleucine	+	0.019	–	–	–	–	–	–	–	–
Leucine	–	–	+	0.164	–	–	–	–	+	0.042
Methionine	+	0.054	+	0.098	+	0.742	+	0.241	+	0.312
Phenylalanine	–	–	–	–	+	0.064	–	–	–	–
Proline	+	1.210	+	1.463	+	0.948	+	2.106	+	0.438
Tyrosine	–	–	–	–	–	–	+	0.226	–	–
Unidentified	2	0.106	1	0.056	1	0.201	1	0.108	1	0.058

ceae showed a relatively lower amount of free amino acid in comparison to the other families ranging between 1.16 and 2.10%.

The free amino acid composition as revealed by TLC has been summed up in Tables 2–9. The number of amino acids present in free form varied from five (*Adhatoda zeylanica*) to 11, as observed in *Antirrhinum majus*, *Bacopa monnieri*, *Barleria cristata*, *Convolvulus arvensis* and *Cuscuta reflexa*. Proline was present as a major amino acid in the pollen of almost all investigated taxa. This is in confirmation with the earlier reports of Pfahler and Linskens[24], and Stanley and Linskens[25], as well as our reports[5,9,20,23]. Alanine, amino-*n*-butyric acid, aspartic acid, glutamic acid, methionine, phenylalanine and others represent some of the other predominant forms. Considerable amount of homology was observed in the amino acid composition of plants belonging to the same family, the homology being more pronounced within plants belonging to the same genus. According to Miflin and Lea[29], amino acids like arginine in certain pollen samples may have a role in storage and transport, while increased levels of amino-*n*-butyric acid reflect the intensity of decarboxylation of glutamic acid[25]. Glutamic acid is, however, a common substrate of glutamine, arginine and proline, and the primary NH_4^+ accepter as well as a product of ammonia assimilation[29]. Except for all the members of Solanaceae and a few other members of the different families, arginine was generally absent in most pollen samples or sometimes present in trace amounts. Thus, the reason behind the accumulation of proline in almost all the examined pollen samples with simultaneous absence of arginine in most cases could be due to the competition

Table 9. Free amino acid composition of pollen of five members of Verbenaceae

	Clerodendrum phlomoidis		*Clerodendrum petasites*		*Clerodendrum splendens*		*Lantana camara*		*Vitex negundo*	
Amino acid	A	B	A	AB	A	AB	A	AB	A	B
Alanine	–	–	+	0.721	–	–	–	–	+	0.122
Arginine	–	–	–	–	–	–	+	0.468	+	0.133
Aspartic acid	–	–	–	–	+	0.683	–	–	+	0.016
Cysteine	–	–	–	–	+	0.841	–	–	–	–
Glycine	+	1.209	+	0.763	–	–	+	0.882	+	0.076
Histidine	+	0.963	+	0.682	+	0.668	–	–	–	–
Lysine	–	–	–	–	–	–	–	–	+	0.048
Methionine	+	0.863	+	1.342	+	0.083	+	0.928	+	0.102
Proline	+	3.121	+	2.785	+	2.108	+	2.360	+	1.634
Threonine	+	0.763	–	–	+	0.092	+	0.532	–	–
Tyrosine	+	0.021	–	–	–	–	–	–	+	0.094
Valine	+	0.382	+	0.038	+	0.086	–	–	+	0.035
Unidentified	1	0.421	2	0.036	1	0.088	2	0.432	–	–

for substrate of the enzymes in the arginine and proline biosynthesis, the accumulation of the products of which depends on a delicate balance of enzyme activity and substrate availability[30]. The presence and activity of the enzyme glutamic dehydrogenase on the other hand, influences the level of amino-*n*-butyric acid formed, while the reductases could influence the level of proline formed from glutamate. Thus, according to Stanley and Linskens[25], modified levels of free amino acids, particularly those occurring in low amounts, may offer a tool for detecting the expression of gene-controlled enzyme synthesis in non-lethal plant mutations. High levels of histidine in the pollen of plants like the three species of *Clerodendrum* may be accounted for due to a gene block of purine metabolism. As suggested by Stanley and Linskens[25], for some sterile variety of apples, this high level of histidine may be due to rechannelling through imidazole glycerophosphate or a failure to incorporate histidine into the protein. Lysine, an uncommon amino acid, is present only in trace amounts in the pollen of *Heliotropium arborescens* and *H. ovalifolum* of Boraginaceae, *Convolvulus arvensis* of Convolvulaceae, *Anisomeles indica*, *Leucas aspera*, *Ocimum sanctum* and *Salvia splendens* of Labiatae and *Vitex negundo* of Verbenaceae. Very little is known about the synthesis of lysine in higher plants[18], but there are reports that increase in free lysine, arginine and valine results in decreased viability of pollen[25]. Valine is also, however, present in very low amounts in all the investigated plant taxa. Apart from these, certain other amino acids were also present in the pollen samples of some species, which could not be identified from the standard amino acids and were categorized as the unidentified types. These may be one among the various unusual amino acid-like compounds found in pollen, as has been reported earlier by Stanley and Linskens[25].

The free amino acid composition of the different members of Acanthaceae (Table 2) reveals a total of 17 amino acids, among which amino-*n*-butyric acid, glycine, proline and valine were present in all the members. Aspartic acid and methionine were also present in all members, except *Adhatoda zeylanica*. Leucine, ornithine and threonine were present only in *Ruellia tuberosa*. The chromatograms in the case of Bignoniaceae revealed 19 identifiable spots with the maximum number present in *Jacaranda acutifolia* and *Kigelia pinnata* (Table 3). Amino acids like amino-*n*-butyric acid, aspartic acid, glutamic acid, phenylalanine, proline and tyrosine were recorded almost in all pollen samples. *Pyrostegia venusta*, however, exhibited a total lack of proline but the presence of hydroxyproline in appreciable amounts. *Jacaranda acutifolia* differed from the other members with the presence of isoleucine and ornithine. Methionine was present only in *Kigelia pinnata*, while threonine and tryptophan were present in *Pyrostegia venusta* only.

In Boraginaceae (Table 4), considerable amount of homology in the amino acid composition was exhibited by the three species of *Heliotropium*. Amino-*n*-butyric acid, aspartic acid and proline were common to all the five members, while alanine, histidine, lysine, tryptophan, tyrosine and valine were the other major amino acids. Glycine was recorded in *Heliotropium indicum* only. Similar homology was also observed in the families of Convolvulaceae and Labiatae (Tables 5 and 6). Convolvulaceae recorded a total of 17 amino acids, with amino-*n*-butyric acid, aspartic acid and phenylalanine common in all the members and alanine, leucine, proline, tyrosine and valine being the other common amino acids present in at least four members. *Cuscuta reflexa* differed from the other four members by the presence of cysteine, glycine, hydroxyproline and tryptophan which were altogether absent from the pollen of *Argyreia nervosa*, *Convolvulus arvensis*, *Ipomoea fistulosa* and *I. pes-tigridis*. This conforms with the classification of Takhtajan[31,32], who separated the family Cuscutaceae not only because of its parasitic nature, but also because of its distinct embryological features, undifferentiated filiform embryo

Table 10. Comparative free amino acid composition of pollen of various members of eight families

Amino acid	Acanthaceae	Bignoneaceae	Boraginaceae	Convolvulaceae	Labiatae (=Lamiaceae)	Scrophulariaceae	Solanaceae	Verbenaceae
Alanine	+	–	+	+	+	+	+	+
Amino-*n*-butyric acid	+	+	+	+	–	+	+	–
Arginine	–	+	+	+	+	–	+	+
Aspartic acid	+	+	+	+	+	+	+	+
Cysteine	–	–	+	+	+	+	+	+
Glutamic acid	+	+	–	–	–	+	+	–
Glycine	+	–	+	+	+	–	–	+
Histidine	+	+	+	–	–	+	+	+
Hydroxyproline	–	+	–	+	–	+	–	–
Isoleucine	–	+	–	–	–	+	+	–
Leucine	+	–	–	+	–	+	+	–
Lysine	–	–	+	+	+	–	–	+
Methionine	+	+	–	–	+	+	+	+
Ornithine	+	+	–	–	–	+	–	–
Phenylalanine	+	+	–	+	–	+	+	–
Proline	+	+	+	+	+	+	+	+
Serine	+	+	–	–	–	+	–	–
Threonine	+	+	–	–	+	+	–	+
Tryptophan	+	+	+	+	–	+	–	–
Tyrosine	–	+	+	+	–	+	+	+
Valine	+	–	+	+	+	–	–	+

Table 11. Pairing affinity values (%) between the eight families based on results of free amino acids of pollen

	Acantha-ceae	Bignonia-ceae	Boragina-ceae	Convolvula-ceae	Labiatae (=Lamiaceae)	Scrophulariaceae	Solanaceae	Verbenaceae
Acanthaceae	100							
Bignoniaceae	57.89	100						
Boraginaceae	40.00	40.00	100					
Convolvulaceae	45.00	38.10	73.33	100				
Labiatae (Lamiaceae)	38.89	26.32	57.14	50.00	100			
Scrophulariaceae	68.42	77.78	42.90	47.00	28.57	100		
Solanaceae	47.37	55.55	44.44	50.00	35.29	61.11	100	
Verbenaceae	42.11	40.00	73.33	52.94	76.93	40.00	47.06	100

and copious endosperm[33]. Earlier, Hutchinson[34] and Lawrence[35] included the genus *Cuscuta* in Convolvulaceae. Many authors split up this family on the basis of parasitic habit of *Cuscuta.* Tiagi[36] had also justified the separation of Cuscutaceae on embryological basis. The homology in amino acid composition was very distinct in the two species of *Ipomoea*, with alanine, amino-*n*-butyric acid, arginine, aspartic acid, leucine, phenylanine and tyrosine common to both. In Labiatae, apart from aspartic acid, glycine, methionine and proline, arginine was common in all members, except in *Anisomeles indica.* Lysine was absent only in *Hyptis suaveolens*, while valine was absent only in *Leucas aspera.*

Apart from Bignoniaceae, Scrophulariaceae also showed the highest number of identifiable amino acids (Table 7). Besides the presence of proline in all the members, hydroxyproline was also present, but in trace amounts in *Bacopa monnieri* and *Digitalis purpurea.* This family showed the presence of certain uncommon amino acids like histidine and serine in *Antirrhinum majus* and ornithine in *Lindenbergia indica.* Leucine was also present in trace amounts in *Digitalis purpurea.* Similar homology was also observed within the various members of Solanaceae and Verbenaceae, with the highest degree of similarity observed between species belonging to the same genus of *Solanum* and *Clerodendrum* (Tables 8 and 9).

The results of pairing affinity between the eight families based on the results of free amino acid analysis have been summarized in Tables 10 and 11. As amino acid composition varies greatly with storage and handling patterns, the data were analysed to study the evolutionary relationships as precautionary measures were taken during harvesting all pollen to avoid such variation. Since the pollen of plants belonging to the same family has more than one amino acid in common and these amino acids are of independent genetic origin, the probability of common ancestry of species of the same family becomes almost a certainity. Since considerable amount of homology

in the amino acid composition was also observed between the families (Table 10), it was assumed that a morphological convergence could be accompanied by chemical convergence or may even bridge the gap from one genus to another, or from one family to the other.

Thus, the application of comparative free amino acid composition to taxonomic groups of different families and determination of the similarity index reveals the highest degree of pairing affinity between Scrophulariaceae and Bignoniaceae (77.78%) followed by Verbenaceae and Boraginaceae (73.33%), and between Convolvulaceae and Boraginaceae (73.33%). Hence Scrophulariaceae and Bignoniaceae are the most closely related, as also Verbenaceae and Labiatae. These results support the classification of Bessey[37], Cronquist[38,39] and Takhtajan[31] in the former, and Bentham and Hooker[40], Bessey[37], Hutchinson[34], Cronquist[38,39] and Takhtajan[31,32] in the latter case. Bessey[37] treated Bignoniaceae as a member of Scrophulariales and derived it from Scrophulariaceae, while Cronquist[38,39] who placed Bignoniaceae in Scrophulariales, pointed out that the two families – Scrophulariaceae and Bignoniaceae are closely related, although the former is dominated by herbaceous members. Similarly, Takhtajan[31] reported that Bignoniaceae is very close to Scrophulariaceae, especially to the tribe Scrophularieae, and probably had a common origin. On the other hand, Bentham and Hooker[40] and Bessey[37] retained Verbenaceae and Labiatae in the same order Lamiales, because of certain resemblances in their corolla and gynoecium. Although Hutchinson[34] also retained Verbenaceae, Labiatae and Phrymaceae in his Lamiales, later, in 1948, he erected a separate order Verbenales, deriving it from Rubiaceous stock. According to him, though in many classificatory systems, Lamiaceae (= Labiatae) and Verbenaceae are placed together, neither has evolved from the other. Both the families represent a climax of evolution as the stem, leaves, inflorescence and gynoecium in the two families are rather similar in their respective groups. Cronquist[38,39] who also placed Verbenaceae in Lamiales along with Labiatae and Phrymaceae, found it rather difficult to draw the line of distinction between the two families, and assumed that the Verbenaceae and Labiatae are the primitive and advanced segments of the same family. Further, Takhtajan[31,32] also included Verbenaceae in Lamiales embracing the families Verbenaceae, Lamiaceae and Callitrichaceae. The similarity of Boraginaceae with Verbenaceae is consistent with the classificatory system of Cronquist[38,39], who considered Boraginaceae to be a single family and attached importance to its resemblance with Verbenaceae and Labiatae. Based on morphology, he had proposed that the gynobasic style and the four nutlet fruit in all the three families have evolved from the unlobed gynoecium, with a terminal style that ripens into a four-seeded drupe. He further stated that if Verbenaceae and the more primitive Boraginaceae were to disappear, the characteristic gynoecium of Labiatae and the rest of Boraginaceae could have originated from a common ancestor. The results of pairing affinity however reveal Boraginaceae to be more closely related with Verbenaceae (73.33%) than with Labiatae (54.14%). The high similarity index between Convolvulaceae and Boraginaceae (73.33%) is supported by Mitra *et al.*[41], according to whom Convolvulaceae bears relationship with Boraginaceae, Hydrophyllaceae, etc. Boraginaceae and Convolvulaceae were however found to be distantly related to Bignoniaceae, Scrophulariaceae and Acanthaceae. Acanthaceae in turn showed a high degree of pairing affinity with Scrophulariaceae (68.42%) followed by Bignoniaceae (57.89%). Its affinity with Scrophulariaceae is in agreement with the view of Bessey[37], who treated it as the most advanced amongst the members of Scrophulariales. This view was further supported by Bensen[42], Cronquist[38,39], Dahlgren[43–46] and Stebbins[47] who retained this family in the order Scrophulariales based on its relationship with the family Scrophulariaceae. Takhtajan[31,32] too reported it to be closely related to the tribe Scrophularieae of family Scrophulariaceae.

The dendrogram constructed on the basis of the free amino acid data (Figure 1) places Solanaceae between Convolvulaceae and Acanthaceae, although it shows higher degree of divergence from Acanthaceae (45.00%) but greater percentage of similarity with Scrophulariaceae (61.11%). This is because Acanthaceae shows higher degree of pairing affinity with Scrophulariaceae (68.42%) than Solanaceae (61.11%), while both the families are distantly related with Bignoniaceae on one hand, and Labiatae, Verbenaceae and Boraginaceae on the other. The results of high percentage similarity of Solanaceae with Scrophulariaceae support the contention of Varghese[48] and Takhtajan[31], according to whom Solanaceae

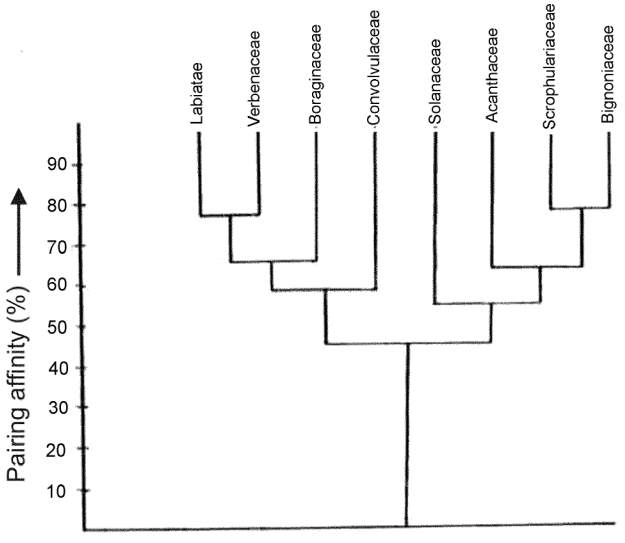

Figure 1. Dendrogram representing average linkage relationship between the eight families as revealed by the free amino acid composition of pollen.

is closely related to Scrophulariaceae, and that various floral features are shared by the two families. However, embryologically, the two families are very distinct and might have had a common origin, but Scrophulariaceae has specialized more than Solanaceae. Takhtajan[31], apart from stating the similarities between Solanaceae and Scrophulariaceae, has also stated the affinities of Solanaceae with Convolvulaceae of the Polemniales. According to him, Solanaceae and Convolvulaceae probably had a common origin from Loganiaceous stock. This view is supported by data on free amino acid composition, which showed the high percentage of pairing affinity between the two families (50.00%). Solanaceae, however, showed greater divergence from Boraginaceae (44.44%), Verbenaceae (47.06%) and Labiatae (35.29%). The results thus support the contention that families which differ by their ecological and morphological attributes can also be placed into discrete groups based on the biochemical and taxonomic characteristics of the free amino acids of the pollen.

Conclusion

Thus it can be concluded that the study of such biochemical characters has laid the foundation for further research on the genetically diverse groups of organisms, which has exemplified a more definitive approach than morphological observation when dealing with similar species or families. The eight families studied can be placed into two categories or clusters based on free amino acid analysis of pollen. Labiatae and Verbenaceae are closely related and should be retained in the order Lamiales, as they can be derived from a common ancestral stock[38]. Bignoniaceae should be best placed in Tubiflorae, near the family Scrophulariaceae. Acanthaceae is also closely related to Scrophulariaceae and an advanced taxon, Solanaceae can be best placed in the order Tubiflorae, close to the family Scrophulariaceae, as there is certainly more resemblance between Solanaceae and Scrophulariaceae than Convolvulaceae. Convolvulaceae also bears some relationship with Boraginaceae which, on the other hand, is more related to Verbenaceae and Labiatae and could have originated from a common ancestor. According to Quicke[49], the characters evolved from one state, i.e. from primitive or plesiomorphous state to another, i.e. the advanced, derived or apomorphous state during evolution. Based on this assumption, he argued that within a group of related organisms in which a character showed more than one state, only one of these can be the ancestral state and the others are the derived (apomorphous) states which have evolved within the group from that ancestral state. This view is strongly supported by the present study. Although, among the phytochemical characters of taxonomic significance, the semantides (protein, DNA and RNA) have been most exploited for getting information on taxonomy and phylogeny, this is the first report of the successful use of free amino acids of pollen to assess the taxonomic significance of isolate groupings. Thus, free amino acids of pollen can also be used as molecular markers. Hence this lays the foundation for future prospects to employ these markers for phylogenetic interrelationships, thus opening the entire biological world for genetic scrutiny and provide access to a nearly unlimited pool of genetic variability which can provide common yardsticks for measuring divergence and facilitate appraisals of evolution.

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