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STUDIES ON PRODUCTION, MORPHOLOGY AND FREE AMINO ACIDS OF POLLEN OF FOUR MEMBERS IN THE GENUS *NYMPHAEA* L. (NYMPHAEACEAE)

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ABSTRACT
The pollen morphology of four species (64 specimens) of the genus *Nymphaea* L. (Nymphaeaceae) was studied and documented in detail using light microscopy (LM), scanning electron microscopy (SEM). The pollen is mostly medium or sometimes small in size, with oblate spheroidal to prolate- spheroidal in shape, monosulcate with granular membranes; the exine is verrucate and psilate and 1.82-3.78µm thick. The present study also deals with pollen production of four species of *Nymphaea* (*N. pubescens*, *N. nouchali*, *N .stellate*, *N. rubra)*. Those are basically high pollen producing plants. Highest numbers of pollen production were showed in *N. pubescens* and lowest in *N. rubra*. The free amino acids composition of these four species also reported in our study with thin layer chromatography. The amino acid content was found to vary from 5.35-7.85% of the total dry weight. Fourteen amino acids were identified, among which glycine, 2-amino –n-butyric acid; β-phenyl alanine and ornithine were present in most pollen samples. The other major amino acids are lysine, glutamic acid, methionine, threonine, valine, alanine, arginine and leucine. Comparative study reveals highest degree of pairing affinities between *N. stellate* and *N. rubra,* followed by *N. nouchali, N. pubescens*.

KEY WORDS: *Nymphaea*, Pollen morphology, Pollen production, Free amino acids, SEM, LM & TLC.

INTRODUCTION

The genus *Nymphaea* Linn*.,* water-lilies is a taxonomically difficult group; different taxonomist distinguish from (Uotila, 2001) up to 12 species (Papchenkov, 2003) of *Nymphaea* spp. L. *Nymphaea* species have high morphological plasticity. Size of leaves and flowers, and also some qualitative characters of flowers, are thought to be strongly dependent on hydrological (especially temperature) and edaphic conditions (Heslop-Harrison 1955, Kupriyanova, 1976; Dubyna ,1982). Floral biology of the genus *Nymphaea* L. was studied by Schneider (1982), Prance and Anderson (1976) and Meeuse and Schneider (1980) on different species of *Nymphaea* mainly focused on protogynous nature and pollination mechanism. *Nymphaea* species can only be distinguished with certainty on the basis of size, shape and exine sculpture of pollen grains (Kupriyanova, 1976; Muntendam *et al*., 1996, Uotila, 2001). Kupriyanova (1976) showed that *Nymphaea* pollen grains were characterized by high morphological stability and could be used for distinguishing species; this opinion is based mainly on the exine sculpture. Interspecific hybrids of *Nymphaea* are characterized by lower fertility (Heslop-Harrison, 1955; Komarov, 1970) and various morphological features of the pollen grains (Kupriyanova, 1976). In contrast to the previous investigations, Poddubnaya-Arnol'dy (1976) noticed that structure, size and shape of pollen grains can vary significantly within one species, although these are diagnostic characters. Pollen is a major source of morbidity for atopic patients, leading to various allergic disorders (Acharya 1980 and Singh *et al*., 1993). Due to various technical difficulties in collecting uncontaminated pollen, critical knowledge regarding pollen biology and biochemistry still remains fragmentary, leading to a serious limitation in the understanding of the physiology and biochemistry of pollen development (Shivanna *et al*., 1979). Pollen grains of Nymphaeaceae are described in literature (Erdtman 1952) as monocolpate except in the genus *Nelumbo.* The grains are bilaterally symmetrical and are generally single aperturate in the other five genera of the family viz., *Brasenia, Cabomba, Eurayle, Nymphaea* and *Victoria.* Studies by Singh *et al.* (1969) in the cultivated varieties of *Nymphaea* have revealed operculate spheroidal and one colpate elongate pollen, and also aperture variance as trichotomocolpate and three porate in small percentage. The monocolpate condition reported in *Nymphaea*has been interpreted as zonisulculate with the coplus encircling grain at its distal end by Erdtman (1952), Walker (1974), and Chanda and Ghosh (1979) or as monoporate aperture aligned to one pole with an operculum by Faegri and Iversen (1975), and Jones and Clarke (1981). Murthy (2000) has described such encircling aperture as zonosulcate in six species of *Nymphaea* in a detailed study on the Nymphaceous members of India. Various ornamentation types such as psilate *(N. nouchali, N. pubescens),* granulate *(N. rubra, N. tetragona),* gemmate *(N. alba)* and baculate (*N. candida)* have been observed by Murthy (2000). 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 characters are excellent taxonomic tool for solving controversial taxonomical and phylogenetic problems. This has been widely recognized all over the world (Mondal *et al*., 1998). There a few earlier works on the use of chemical constituents of pollen in understanding plant affinity and phylogeny (Mondal and Mandal, 1998; Mukherjee and Chanda, 1990). This article also has been focused on the total free amino acids content and free amino acids composition of pollen. This can use as taxonomic markers. Based on the homology in amino acid composition we are trying to resolve the relationship among the selected species. 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 (1970). 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 (Stanley and Linskens, 1974). Total level of amino acids is usually higher in pollen than in the other free explants of the plant. The most abundant free amino acids reported previously 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, valine (Stanley and Linskens 1974) and proline, cysteine, aspartic acid, histidine, glutamic acid, ornithine, amino-n-butyric acid and hydroxyl proline (Mondal and Parui, 2009).

To archive the goals we used a complex approach: analyzing pollen size, exine sculpture and also pollen production, free amino acids composition of fresh plant's pollen their relation from taxonomical view. The present study gives an account of knowledge about pollen morphology, pollen production and pollen phytochemistry (free amino acids) of four common members of the family Nymphaeaceae.

MATERIALS AND METHODS

Pollen grains of the four recognized species (64 specimens and 36 localities has been selected) of *Nymphaea* were collected directly from different aquatic field of South-Eastern part of India. The list of voucher specimens is deposited in the "Vidyasagar University Harbarium" (VUH) Midnapore, India. Table 1 shows the names, collectors, origin and general distribution of the samples under study. Fully mature anthers were removed from the specimens and were prepared by the standard acetolysis method (Erdtman 1960), after which they were mounted in glycerin jelly and sealed with paraffin max for light microscopy (LM). Measurements and morphological observations were made with an Olympus –CH20i BIMU, under E40, 0.65 and oil immersion (E100, 1.25), using a 10x eye piece. For scanning electron microscopy (SEM) acetolysed pollen grains in a 70% ethanol solution were pipetted directly on to aluminum stubs with double sided cellophane tape, and air dried at room temperature under an inverted flask. Specimens were coated with gold-palladium using at 20kV and photographed. The measures were based on 15-20 readings from each specimen. Characteristic of the pollen; pollen diameter (P), equatorial diameter (E) [measured by 400 times magnitude], Colpus length (clg), colpus width (clt), exine thickness (ET), intine thickness (IT), apocolpium and mesocolpium were measured. The terminology used is in accordance with Erdtman (1952), Wodehouse (1935), Blackmore and Persson (1996) and Punt *et al*. (2007). Pollen production study was done by the method of Nair and Rastogy (1963), Mondal and Chandra (1981). Unopened flower buds were collected in the morning. One anther was crushed and dispersed uniformly in 50 drops of glycerin. One drop of dispersion was put on a slide and covered with an 18×18mm cover glass and was counted under light microscope. Then average multiplied by 50 to get the number produced by one anther and finally to get the number produced per flower. Repeat this process 10 times for different anther in different flower randomly in one species individual.

TABLE 1. Names, collectors, provenance and general distribution of species considered for this study.

Taxa	Collectors and Localities	General distribution
*Nymphaea pubescens*Willd.	*D. Bhunia*, India: West Bengal, Paschim Medinipur, Bankura, Murshidabad, Hoogly, Howrah. In aquatic wetland, detaches.	India (endemic from South- eastern India)
Nymphaea nouchali Burm.f.	*D. Bhunia*, India: West Bengal, Paschim Medinipur, Bankura, Murshidabad, Hoogly, Howrah. In aquatic wetland, detaches.	India (endemic from South-eastern India)
Nymphaea stellata Willd.	*D. Bhunia*, India: West Bengal, Paschim Medinipur, Bankura, Murshidabad, Hoogly, Howrah. In aquatic wetland, detaches.	India (endemic from South- eastern India)
Nymphaea rubra Roxb. ex Andrews	*D. Bhunia*, India: West Bengal, Paschim Medinipur, Bankura, Murshidabad, Hoogly, Howrah. In aquatic wetland, detaches.	India (endemic from South- eastern India)

TABLE 2. Comparison of flower morphology of *Nymphaea*species

Plant Taxa	Flower diameter (cm)	Number of petals	Number of stamens	Length of stamen (cm)	Ovary diameter (cm)	Number of ovary chambers	Length of Stigma appendages
N. pubescens	10.35±0.42	17.50 ±1.80	61.50± 0.80	2.5 ± 1.25	1.87 ± 0.06	17.00 ± 0.32	0.31 ± 0.03
N. nouchali	9.60± 0.11	16.80 ± 0.6	58.50± 2.38	2.8 ± 1.23	2.10 ± 0.12	15.75 ± 0.45	0.19 ± 0.02
N. stellata	16.95±1.12	23.08 ± 0.47	56.61±1.31	3.3 ± 0.17	0.97 ± 0.21	22.03± 0.54	1.36 ± 0.09
N rubra,	19.95±1.12	22.18±0.67	54.90±2.22,	2.5 ± 1.08	1.77 ±0.11	22.40 ±0.59	1.11 ± 0.07

TABLE 3. Flowering period, anthesis time of selected plants in the family Nymphaeaceae

Name of the plant	Mode of pollination	Habit	Flowering period	Time of anthesis
N.pubescens	Entomophilly	Aquatic herb	May-November	Day
N. nouchali	,,	,,	June-January	Day
N. stellata	,,	,,	July- November	Day & Night
N. rubra	,,	,,	Throughout the year	Day & Night

TABLE 4. Pollen production in the four species of *Nymphaea* L. in the family

Plant Name	No. of Anther(mean)	Length of Anther (mm)	Av. No. of pollen/anther	log pollen/anther	Av. No. of pollen/flower
N. pubescens	50	1.14	16857	4.227	842850
N. nouchali	50	1.25	14512	4.161	725600
N. stellata	50	1	13983	4.145	699150
N. rubra	50	1.32	11461	4.056	573050

TABLE 5. Pollen characteristics of the examine taxa. Marks (except variations) are in micrometers (µm)
Variation numbers are bar numbers in LM. *M* Arithmetic mean, *sd* standard deviation, *var* variations, *P*- polar diameter, *E* -equatorial diameter, *P/E*- pollen shape, *Ex*- exine thickness, *Ex/Int* the ratio of exine to intine thickness, *clg* colpus length, *clt* colpus width, *sculpture* ornamentation

Taxa		P	E	P/E	Ex	Ex/Int	clg	Clt	Sculpturing LM and SEM
N. pubescens	M	33.280	32.170	1.034	≅ 3	≅ 3/2	22.989	3.383	verrucate, wart-like element, broad than high in one side; or sometime psilate, pollen wall with smooth surface in other side.
	Sd.	±1.931	± 1.863	Prolate-spheroidal			± 1.845	±0.947	
	Var.	11-14	9-11				15-24	1-3	
N. nouchali	M	37.601	38.087	0.987	≅ 3	≅ 3/2	22.588	3.851	fossulate with irregularly shaped grove in the surface of pollen wall
	Sd.	±2.693	±2.043	Oblate-spheroidal			±1.40	±0.584	
	Var.	13-16	10-12				18-22	8-10	
N. stellata	M	31.672	32.446	0.976	≅4	≅4/2	23.153	4.317	verrucate, wart-like element, height ≤ width; sometimes granulate, scale like structure.
	Sd.	±2.76	±3.24	Oblate-spheroidal			±1.22	±0.714	
	Var.	16-17	12-15				17-23	2-4	
N. rubra	M	17.324	17.324	1.00	≅2	≅2/1	15.262	4.509	psilateorscabrate, pollen wall with smooth surface
	Sd.	±0.540	±0.431	Spheroidal			±1.031	±0.543	
	Var.	15-18	13-16				6-9	5-6	

The pollens were collected separately from at least 64 different individual plants of the 4 selected species and qualitative analysis of free amino acids. Each sample collected, *i.e*. 20 data for each species to know the amino acid composition. The data of total amino acid content represented in Table (6 and 7) 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 (1996). 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 vortexes for 2 min, and then centrifuged at 900 *g* for 10 min. The pellet was re-extracted with 2 ml of methanol: chloroform: water, vortexes 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 ninhydrinand 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 followed the method Sadasivam and

Manickam (1992). 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 aluminum sheets (Merck) were used for performing TLC, according to the method described by Sadasivam and Manickam (1996). 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 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 (1963) and Romero Lopes *et al.* (1979) was used to analyses the data of free amino acid composition.

Pairing affinity,(PA) = [Amino acid common to the species A and B] / [Total amino acids in A and B] ×100

TABLE 6. Total free amino acid content (%) of pollen of four species (data based on ten readings for each species).

Plant	Mean value of total free amino acids content (µmol/mg dry wt.)	Standard deviation
N. pubescens	6.85	0.091
N. nouchali	6.50	0.084
N. stellata	5.35	0.077
N. rubra	7.82	0.093

TABLE 7. Showing comparative free amino acid composition of pollen of four members in the family Nymphaeaceae. Amount in µmol/mg dry wt (data based on mean value of five readings for each species).

Amino acid	*N. pubescens*	*N. nouchali*	*N. stellata*	*N. rubra*
Alanine	0.133		0.883	0.135
Amino-*n*-butyric acid	1.230			0.668
Arginine			0.542	2.785
Aspartic acid				
Cysteine				
Glutamic acid	0.563			
Glycine	1.041	1.648		
Histidine				
Hydroxyproline				
Isoleucine				
Leucine	0.480		1.342	
Lysine	1.106			
Methionine	0.312			
Ornithine		1.210	2.108	2.080
Phenylalanine	2.106	2.116		
Proline				
Serine				
Threonine		1.463	1.207	
Tryptophan	1.202			
Tyrosine				
valine		0.106	0.193	0.263

TABLE 8. Pairing affinity values (%) among the four members of Nymphaeaceae (based on results of free amino acids of pollen).

Taxa	*N. pubescens*	*N. nouchali*	*N. stellata*	*N. rubra*
N. alba	100			
N. nouchali	16.67	100		
N. stellate	14.28	37.5	100	
N. rubra	16.67	25	57.15	100

RESULTS AND DISCUSSION

Flower morphology

In *N. rubra* flower diameter (cm) is 20.03 ±2.10, number of petals 24.54 ± 1.98; number of stamens 53.89 ± 1.23, length of stamen (cm) 2.5 ± 1.08, ovary diameter (cm) 1.73 ± 0.10, number of ovary chambers 21.87 ± 2.09, length of stigma appendages (cm) 1.09 ± 0.09 whereas *N. stellata* flower diameter (cm) is 18.07 ±1.13, number of petals 24.45 ± 1.77; number of stamens 55.23 ± 1.02, length of stamen (cm) 3.3 ± 0.17, ovary diameter (cm) 1.02 ± 0.41, number of ovary chambers 21.09± 1.59, length of stigma appendages (cm) 1.86 ± 0.08. Flower morphology of *N. pubescens* flower diameter (cm) 12.71 ± 1.25, number of petals 19.30 ±0.97, number of stamens 59.94 ± 1.70, length of stamen (cm) 2.5 ± 0.98, ovary diameter (cm) 1.92 ± 0.08, number of ovary chambers 18.13 ± 1.30, length of stigma appendages (cm) 0.53± 0.05 and in *N. nouchali* flower diameter (cm) 11.60 ± 0.09, number of petals 17.35 ±0.80, number of stamens 59.80 ± 1.32, length of stamen (cm) 2.8 ± 1.23, ovary diameter (cm) 2.03 ± 0.02, number of ovary chambers 17.24 ± 1.20, length of stigma appendages (cm) 0.49± 0.05 (Table -2). On the taxonomy basis flower morphology of *N. stellate* and *N. rubra* are less different in micro-morphological characters and also flower morphology of *N. pubescens* and *N. nouchali* are morphologically more related than the other species of *Nymphaea.* The flower morphology of *Nymphaea*species is more or less similar to the observations of Mitra (1999) and Hossain *et al.* (2000).

Pollen production

Pollen grains of the aquatic plants affect the water environment. The selection of aquatic plants was made on the basis of abundance in the locality. In the present investigation number of pollen grains per anther per flower was estimated. Total four aquatic rooted hydrophytes with floating leave plants i.e. *N. pubescens*, *N. nouchali*, *N. stellata*, *N. rubra* were studied. Among these aquatic taxa *N. pubescens* produced highest numbers of pollen grain (742850) per flower while lowest numbers of pollen showed in *N. rubra* (Table 3–4 & Fig.1). Anthes is showed great variation among different species of *Nymphae. N. pubescens* showed anthesis for three consecutive nights, *N. rubra* for four consecutive nights and in *N. nouchali* it was for three consecutive days. In all the species anthesis for the second time was found to take place about half an hour earlier. The number of flower of a taxon is always variable due to ecological condition. Opening and closing time of flowers were very much influenced by the intensity of sunlight and hence temperature also. According to Prance and Anderson (1976) temperature was more effective than sunlight for the opening and closing of *Nymphaea* flowers. The present investigation showed that highest no. of pollen grain produce in *N. pubescens* (842850 no. of pollen per flower) than the other three species and lowest number of pollen produced in *N. rubra*. After the study it has been concluded that pollen production in aquatic hydrophytes were not related directly with the size of flower and the anther or size of pollen grains but it also controlled by other factors such as periodicity, response to light and nutrient availability. Molina *et al* (1996) find that if the size of pollen and length of the anther is small then the numbers of pollen produced by particular taxon is lowest. It has been observed that the rooted hydrophytes with floating leaves produced highest amount of pollen grains than the submerged, marginal and free floating hydrophytes (Mondal and Mandal 1997). Our investigation also supports these previous observations. Due to self-pollination (Entomophilly) pollen grains production is high in the genus *Nymphaea*.

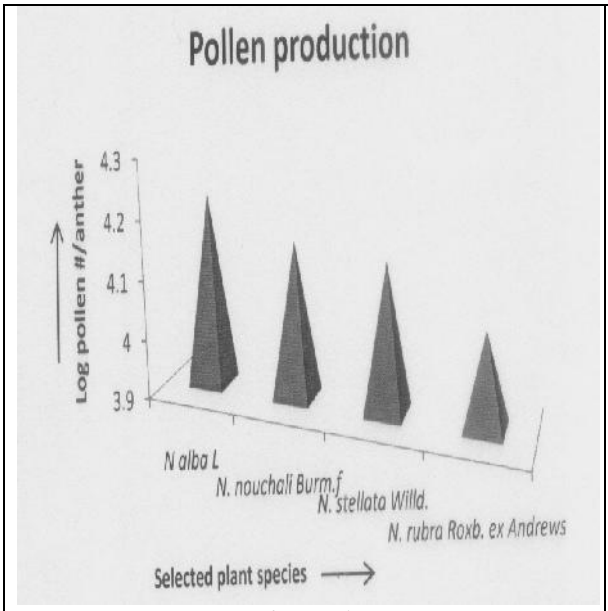


Figure- 1

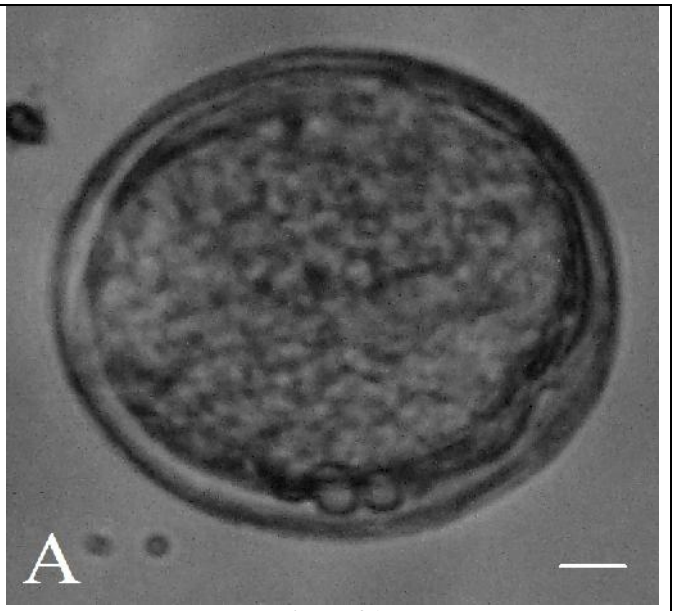
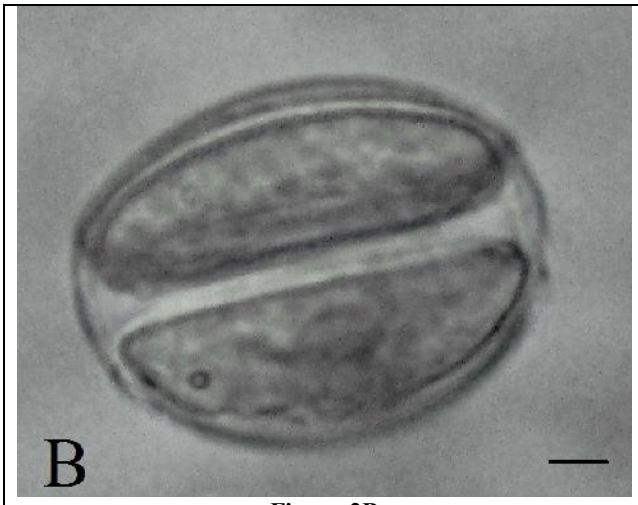


Figure-2A



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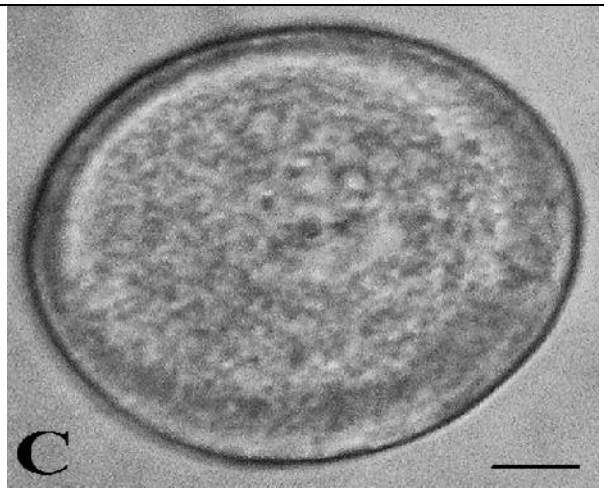

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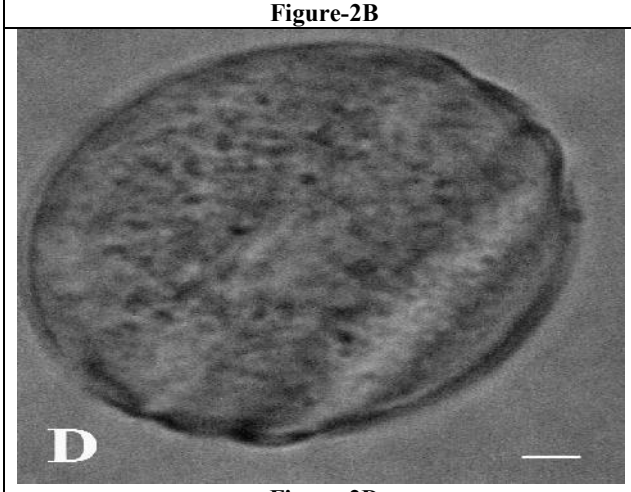

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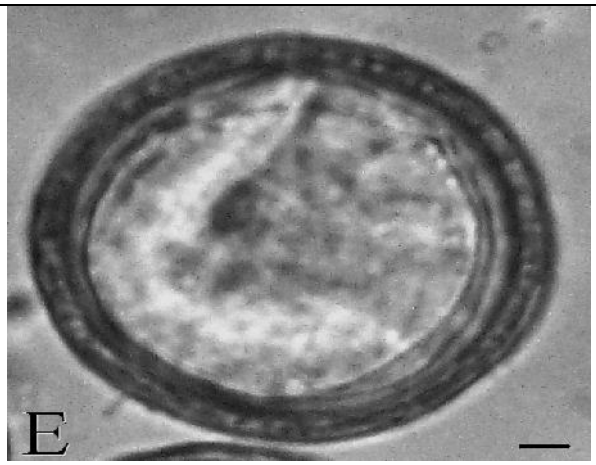

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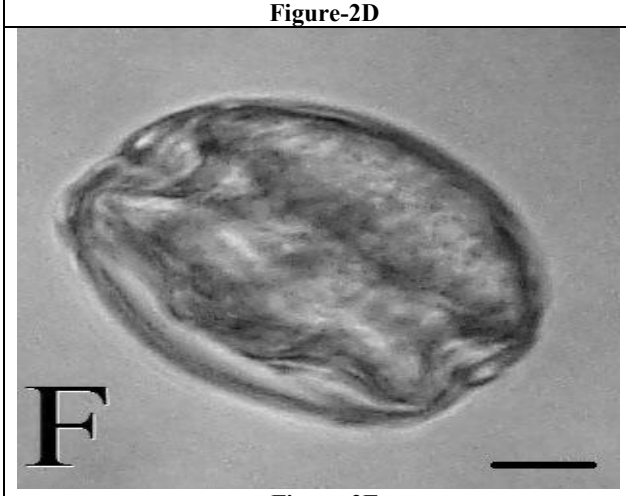

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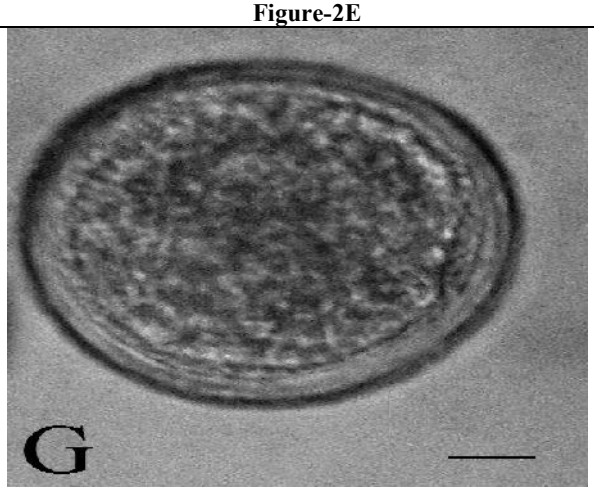
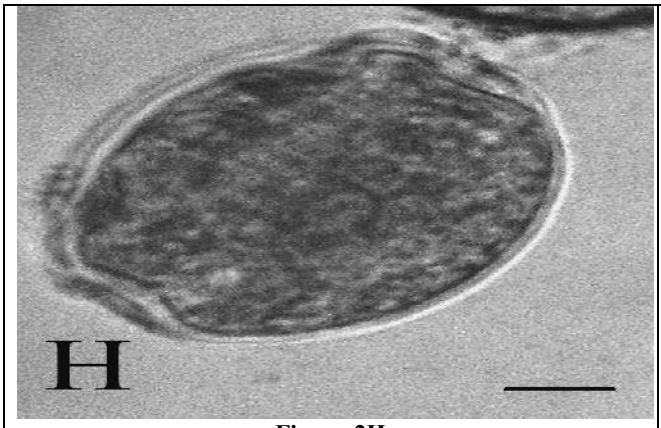


Figure-2G

Pollen Morphology

Nymphaea pubescens (Fig. 2E, F & Fig. 3Ia, 3I-J).− Pollen types are bilateral, heteropolar. Pollen unit is monad, dispersal unit consisting of single pollen grains. Pollen class is inaperturate and shape and size is prolate-spheroidal (P/E: 1.034). Pollen dimension is medium size (Polar diameter × Equatorial diameter = 33.280 × 32.170 µm) and ring-like aperture, circumferential aperture (situated more or less equatorially); angular perturate, pollen grain with an angular outline, thickening at the round of aperture membrane. The outline of pollen is in equatorial view circular to elliptic-obtuse convex; polar view triangular to circular or obtuse convex. The exine ornamentations of pollen are verrucate, wart-like element, broad than high in one side; or sometime psilate, pollen wall with smooth surface in other side. The exin and intine ratio is 3: 2. Exine is 3.086µm thicker (Table-5). *Nymphaea nouchali* (Fig. 2C, D & Fig. 3K, L). − Pollens

arebilateral, heteropolar type. Pollen unit is monad, dispersal unit consisting of a single pollen grain and pollen class is inaperturate, monocolpate; polymorphic. Shape and size of the pollen is oblate-spheroidal Polar diameter/Equatorial diameter: 0.987; some time outline, pollen in-folding (dry pollen).



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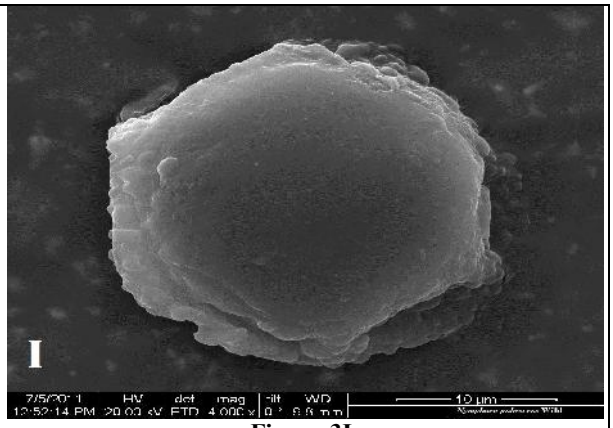


Figure-3I

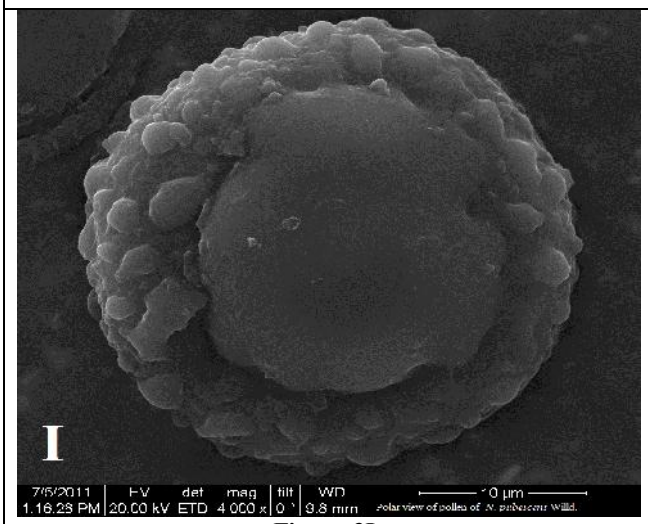


Figure-3Ia

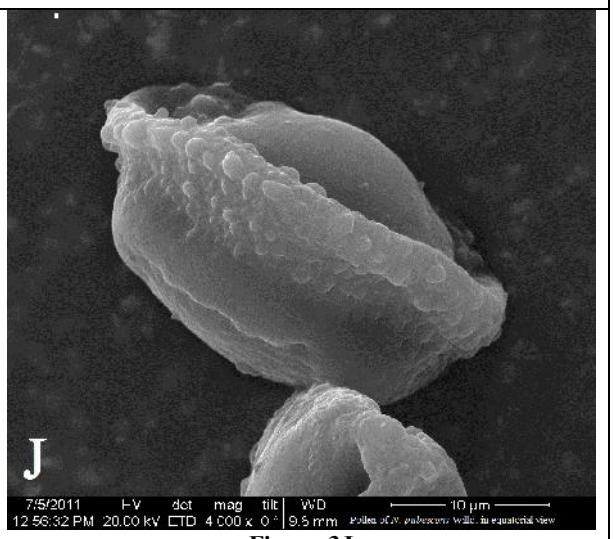
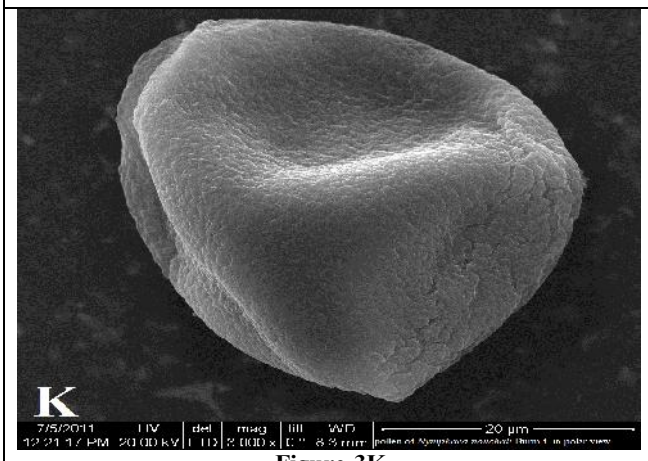


Figure-3J



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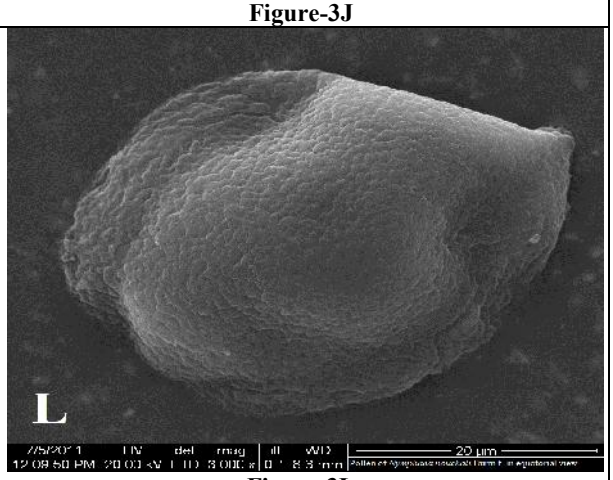
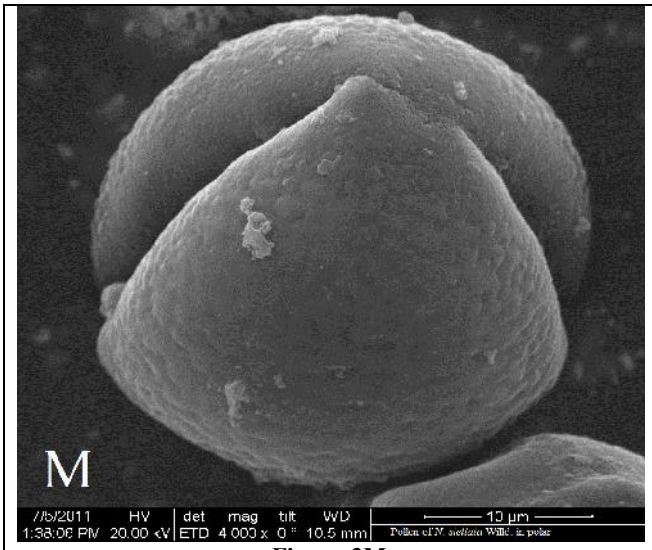


Figure-3L



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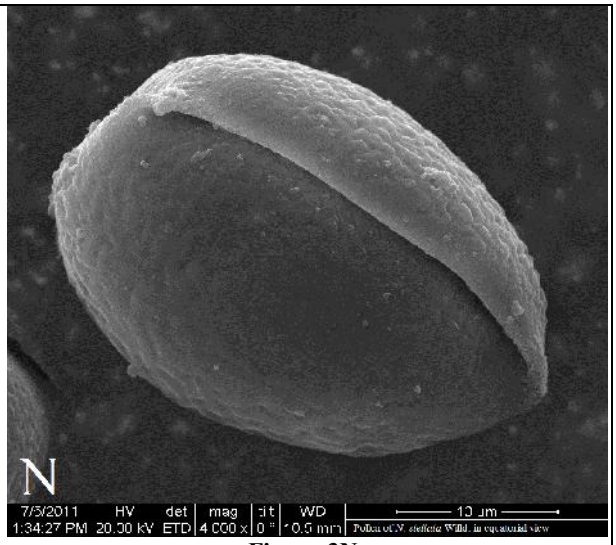

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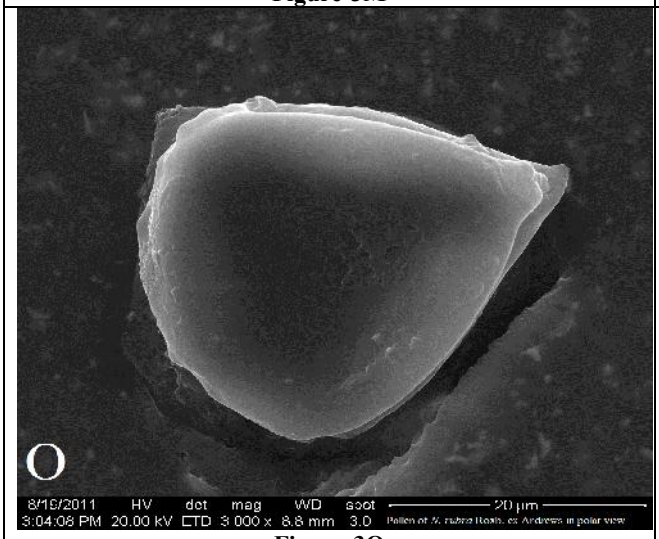

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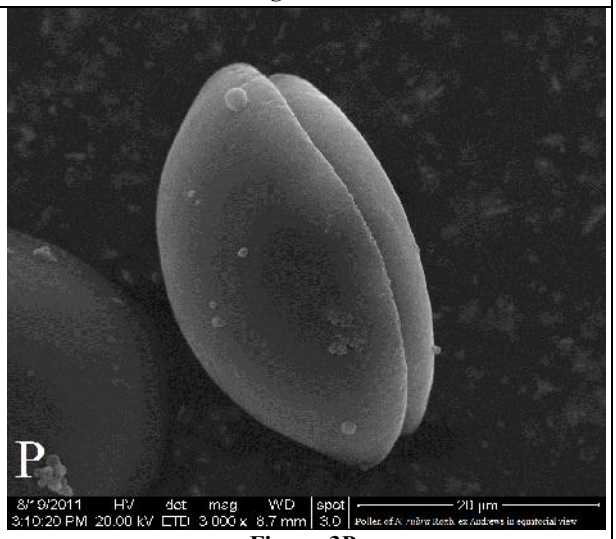

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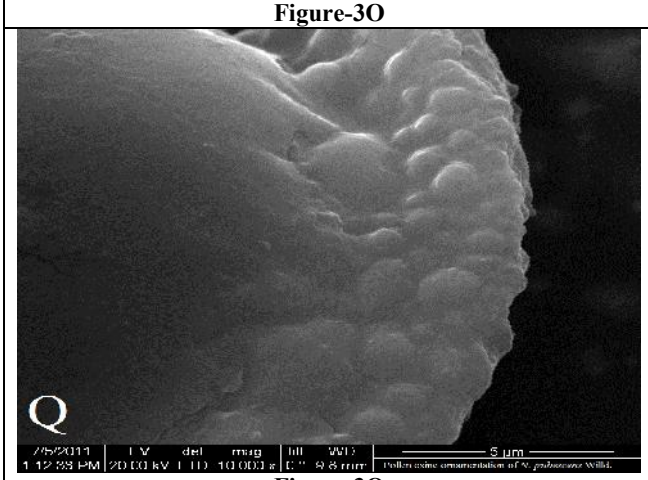


Figure-3Q

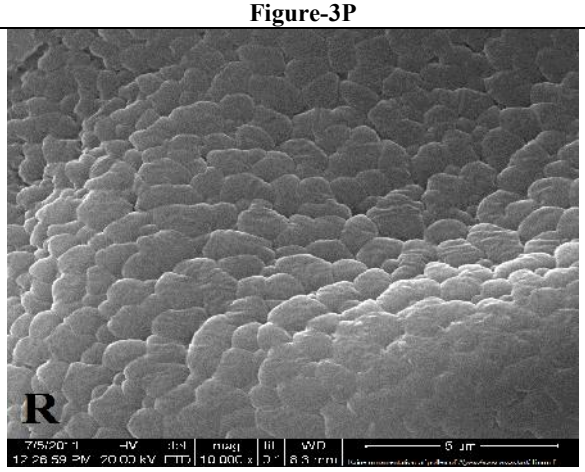


Figure-3R

The dimension of pollen is medium size (P×E= 37.601×38.087µm). Aperture of the pollen is tricolpate, pollen grain with colpi: long, straight, wide, shallow, with obtuse ends, colpus length 22.558µm, colpus wide 3.851µm. Outline of the pollen is in equatorial view circular to elliptic-obtuse convex; polar view triangular to circular-obtuse convex, sometime circular. Exine ornamentation of pollen is Verrucate grains with equatorially oriented ridges or radially oriented looped ridges or fossulate with irregularly shaped grove are olatesurface on the pollen wall. The exin and intine ratio is 3:2. Exine is 2.96µm thicker (Table 5).

Nymphaea stellata (Fig. 2A, B & Fig. 3M, N). −Pollen type is bilateral, heteropolar and unit ismonad, dispersal unit consisting of a single pollen grain. Pollen class is disulcate. Shape and size of pollen spheroidal, Polar diameter/equatorial diameter = 0.976, boat-shaped and dimension of pollen is medium size (P/E= 31.672×32.446µm). The aperture is sulcate, pollen with two sulci in equatorial view slg: 23.153µm, slt: 2.173µm; ring-like aperture running meridionally; colpi is long, deep, nearly reaching the poles, with acute ends; colpus length 31.306µm, wide 4.317µm. Outline of pollen in polar view circular to triangular-obtuse convex, equatorial view rectangular to elliptical obtuse-convex. Exine ornamentation of pollen verrucate, wart-like element, height ≤ width; sometimes granulate, scale like structure. Exin and intine ratio is 4:2 and exine is3.782µm thick (Table 5)**.**

Nymphaea rubra (Fig. 2G, H & Fig. 3O, P) −Pollen unit is monad, dispersal unit consisting of a single pollen grain. Pollen class is monocolpate (dominant) and megaporate, operculate; dimorphic, disulcate. Shape and size of pollen spheroidal, Polar diameter/Equatorial diameter = 1 and pollen dimension is small size (P×E= 17.342×17.342µm). Aperture of pollen is ring-like, running meridionally, circumferential aperture, sulcate, pollen with two sulci in equatorial view, sulcus length 11.153µm, sulcus wide 1.073µm; colpus margin with elevated structures which are fused, semi fused or free, thus fortifying the colpus edge, colpi: long, deep, nearly reaching the poles, with acute ends; colpus length 15.262µm, colpus wide 3.317µm.Outline of the pollen in polar view triangular-obtuse convex; equatorial view elliptical obtuse-convex. Exine ornamentation of pollen are psilate or scabrate, exine surface plated or tuberculate, being with flat islands or rounded tubercles. The exin and intine ratio of pollen is 2:1and Exine thickness1.823µm (Table 5).

Variations in pollen morphological characters

All the *Nymphaea* species examined are characterized (Table 5) by monad, bilateral, heteropolar medium sized pollen, indicating that the genus *Nymphaea* is a closely related entity. This is in agreement with previous reports (Erdtman, 1952). But in *N. pubescens* and *N. nouchali* pollen class is inaperture, prolate-spheroidal to oblate-spheroidal other than two species. In *N. stellate* and *N. rubra* pollen class is disulcate and spheroidal in size and shape. Sizes of pollen grains for *Nymphaeapubescens*, *Nymphaearubra* and *Nymphaea nouchali* overlap considerably; moreover, this character show large variation within populations. Pollen morphology is presently a globally accepted tool in consideration of plant taxonomy and evolution, and with the SEM providing information on finer architecture, the application of pollen in comparative morphology has become possible in gaining new knowledge at varietal and even ecosystem levels. The above situation is amply reflected in the pollen morphology of *Nymphaea,* an aquatic plant under the strain of water related stress and environmental conditions. The present report is based on four species of *Nymphaea* together with some ecological variants indicated by colour variations of the flower, and the data generated provide indications of the index value of pollen morphology in the identification and substantiation of species on the one hand and of the colour variations (ecotypes) on the other.

The pollen grains of the analyzed taxa were divided into groups according to their equatorial view and shape as follows:

According to the equatorial view−Type I: elliptic-obtuse convex-*Nymphaea rubra*.Type II: elliptical to rectangular obtuse-convex-*Nymphaea stellata*. Type III: circular to elliptic-obtuse convex- *Nymphaea nouchali*, *Nymphaea pubescens*.

According to pollen shape –Type I: Prolate-spheroidal-*Nymphaea pubescens*, Type II: Spheroidal-*Nymphaea rubra* and *Nymphaea stellata*. Type III: oblate-spheroidal-*Nymphaea nouchali*.

The aperture system-In *N. pubescens* ring-like aperture, circumferential aperture (situated more or less equatorially); angulaperturate, pollen grain with an angular outline, thickening at the round of aperture membrane. In *N. nouchali* aperture system colpate, colpi long, straight, wide, shallow, with obtuse ends. But in *N. stellate* and *N. rubra* both are boat like sulcate aperture, colpi long, deep, nearly reaching the poles, with acute ends.

Exine ornamentation-The ornamentation of *Nymphaea* species was observed psilate or scabrate to verrucate, fossulate with irregularly shaped grove in *N. nouchali* and *Nymphaea stellata*. The surface of pollen wall is smooth in *N. rubra*. Tectate-infrastructure and exine thickness ranged between 1.823µm and 3.782µm. The thickest exine was observed in *Nymphaea stellata* and thinnest exine was observed in *Nymphaea rubra*. Pollen morphology can be useful in supporting taxonomic suggestions (Clark *et al*., 1980). Application of palynology is very diverse and multidisciplinary. However, the role of pollen morphology is of significance in taxonomic debate for classification. Pollen characters have proved useful for systematic purposes in various plant families. Tomsovic (1997) utilized pollen character as additional information for systematic studies. Huang (1972) also used pollen characters for systematic purposes. In the genus *Nymphaea,* pollen shape, apertures, polarity and tectum type exine width, P/E ratio have proved useful diagnostic characters to identify the species of this genus. However, the exine pattern and apertural types are the most significant pollen characters. It is very interesting to note that the most of the aquatic taxa are wind pollinated. Selected aquatic dicots *Nymphaea stellata*, *Nymphaea nouchali*, *Nymphaea pubescens* and *Nymphaea rubra* are pollinated by insects. There is a correlation between pollen morphology and pollination mechanism. A definite relationship is exhibited between pollen characters and pollination types especially in entomophily. Pollen grains of entomophilous taxa are characterized by compound apertures *i.e.,* colporate, prolate- spheroidal shape, generally large, thick-walled, sticky and with dry and with scabrate-areolate tectum, sulcate, fossulate with irregularly shaped grove, verrucate, wart-like element, broad than high in one side; or sometime psilate, pollen wall with smooth surface, ring-like aperture running meridionally. Pollen morphology, in our opinion, has much less diagnostic value than was thought before (Komarov 1970, Kupriyanova 1976, Dubyna 1982, Uotila 2001).

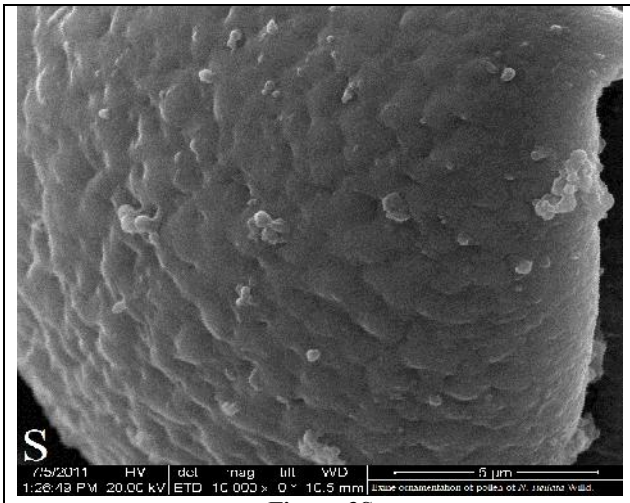


Figure-3S

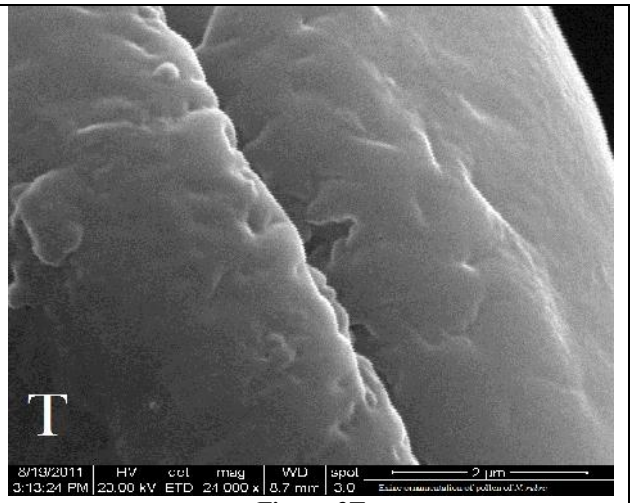


Figure-3T

Only *Nymphaea pubescens* can be distinguished on the base of exine sculpture while *Nymphaea rubra* and *Nymphaea stellata* do not differ on this character, contrary to the findings of researchers who worked with small samples (Kupriyanova, 1976, Muntendam *et al*. 1996). Morphologically pollen grain of *Nymphaea rubra* and *Nymphaea stellate* are more similar in shape, size (Spheroidal), aperture (sulcate, ring-like aperture running meridionally), outlines and ornamentation, followed by *Nymphaea nouchali* which support the strong affinity between them as follow in free amino acid composition of pollen *i.e.* describe in below.

Key to the species of *Nymphaea* on the basis of pollen morphology

1a. Spheroidal P/E = 1, Pollen exine orientation psilate or scabrate, shape triangular-obtuse convex in polar view, exine thickness 1.823µm……*Nymphaea rubra*

1b. Spheroidal P/E= 0.976, Pollen exine orientation verrucate, wart-like element, height ≤ width; sometimes granulate, scale like structure, shape triangular-obtuse convex in polar view, exine thickness 3.782µm ……*Nymphaea stellata*

2. Oblate spheroidal P/E: 0.987, Pollen exine orientation fossulate with irregularly shaped grove in the surface of pollen wall like, exine thickness 2.96µm……*Nymphaea nouchali*

3. Prolate spheroidal P/E: 1.034, Pollen exine orientation verrucate, wart-like element, broad than high in one side; or sometime psilate, pollen wall with smooth surface in other side, exine thickness 3.086µm…….....*Nymphaea pubescens*

Free amino acid analysis of pollen grains

Quantitative analysis

Analysis of free amino acids on dry weight of plant pollen sample extracts are considerably low (below 10%) of the selected plant species and ranged between 5.35% and 7.82%(Table 6). *N. stellate* showed extremely low level of the total free amino acids content *i.e*., 5.35% of the pollen dry weight. *N. rubra* showed the highest level of free amino acids i.e., 7.82% followed by *Nymphaea pubescens* (6.85%) and *N. nouchali* (6.50%). Analysis of free amino acids on dry weight of plant pollen sample extracts are considerably low (below 10%) of the selected plant species and ranged between 5.35% and 7.82%. On this quantitative estimation of free amino acid have minute differences among the taxa. *N. stellate* showed extremely low level of the total free amino acids content of the pollen dry weight. *N. rubra* showed the highest level of free amino acids followed by *N. pubescens* and *N. nouchali*.

Qualitative analysis

The free amino acids are isolated and identified by thin layer chromatography (TLC). The number of amino acids present in pollen as free form condition varied from five (*N. nouchali*.) to seven (*Nymphaea pubescens*). Lysine, methionine, glutamic acid and tryptophane uncommon amino acids are present only in trace amounts in the pollen of *Nymphaea pubescen* and Threonine also an uncommon amino acids are present only in trace amounts in the pollen of *Nymphaea nouchali*. Valine is also, however, present in low amounts in two investigated plant species (*N. nouchali*, *N. rubra*). The free amino acid composition of the different members of Nymphaeaceae reveals a total of 14 amino acids, among which ornithine is present in all the members except *N. pubescence*. Arginine and alanine present only in *N. stellate*, *N. rubra.* β-phenyl alanine is present in *N. stellate* and *N. nouchali* Burm.f. Leucine also present in *Nymphaea pubescens* and *N. stellata*. 2, amino-n-butyric acid is present in *Nymphaea pubescens* and *N. rubra*. Glycine is also identified in *Nymphaea pubescens* and *N. nouchali* (Table 7, Fig. 4).

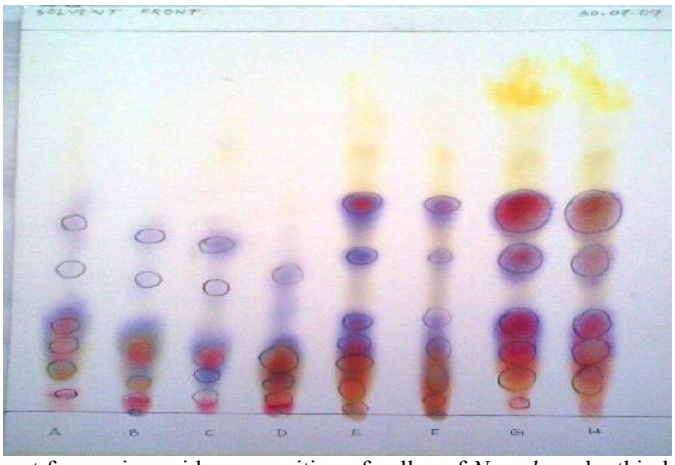


Fig. 4. Showing different free amino acids composition of pollen of *Nymphaea* by thin layer chromatography.

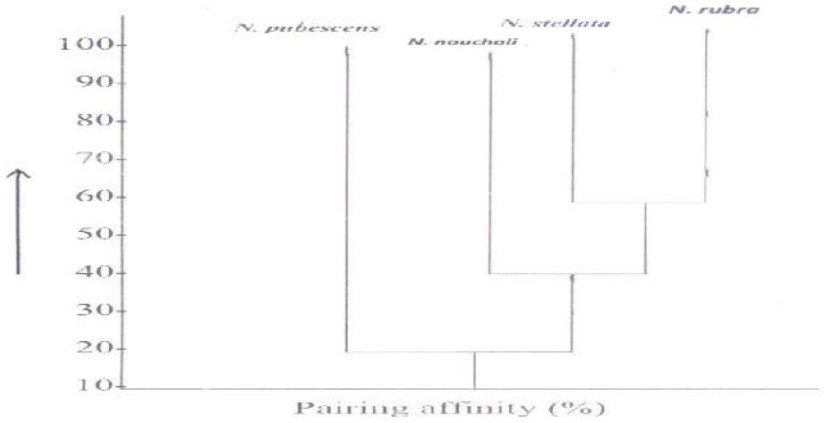
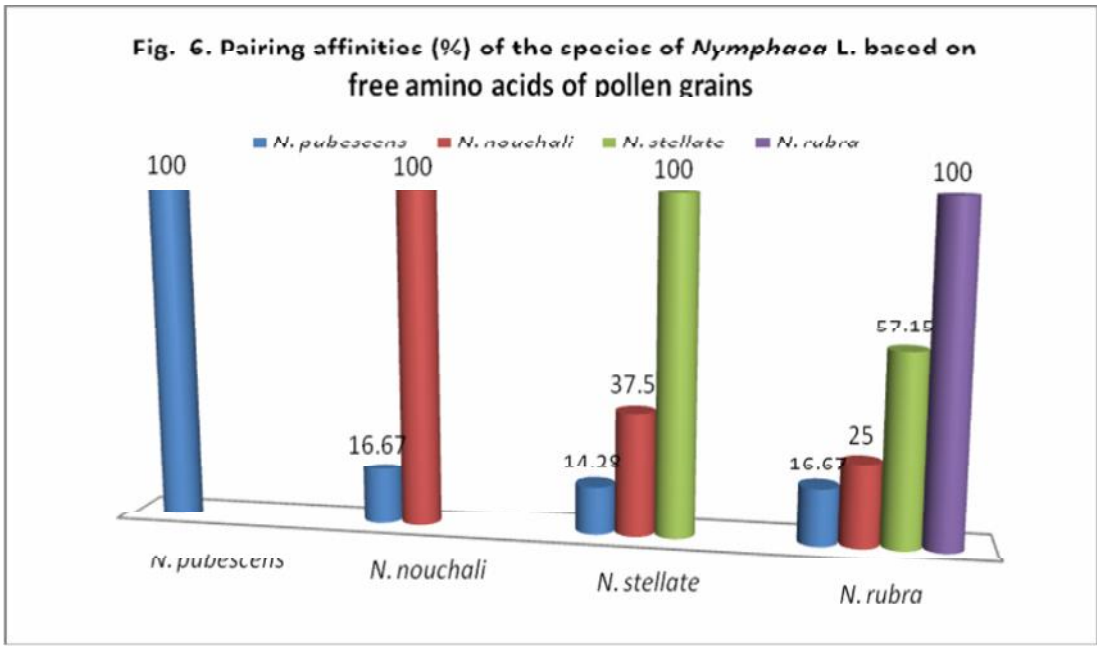


Fig. 5. Dendrogram representing average linkage relationship among the species *Nymphaea* as revealed by the free amino acid composition of pollen.



Considerable amount of homology was observed in the amino acid composition of plants species being to the more pronounced within the investigated genus. According to Miflin and Lea (1977), 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. Glutamic acid is a common substrate of glutamine, arginine and proline and the primary NH_4^+accepter as well as a product of ammonia assimilation. Accumulation of arginine in plant species depends in a delicate balance of enzyme activity with proline (Vance and Zaerr, 1990). Thus, according to Stanley and Linskens (1974), 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. Lysine, methionine, glutamic acid and tryptophane are uncommon amino acids present only in trace amounts in the pollen of *Nymphaea pubescens* and threonine also uncommon amino acids are present only in trace amounts in the pollen of *Nymphaea nouchali*. Very little known about the synthesis of lysine in higher plants (Erdtman, 1963), but there are reports that increase in free lysine, arginine and valine results in decreased viability of pollen (Stanley and Linskens, 1974). Valine is also, however, present in low amounts in two investigated plant species (*N. nouchali*, *N. rubra*). These may be one among the various unusual amino acid-like compounds found in pollen, as has been reported earlier by Stanley and Linskens (1974). The results of pairing affinity between the four species based on the results of free amino acid analysis have been summarized. As amino acid composition varies greatly with storage and handling patterns, the data were analyzed to study the evolutionary relationships as precautionary measures were taken during harvesting all pollen to avoid such variation. Comparative free amino acids composition of four members of Nymphaeaceae reveals highest degree of pairing affinities (Table 8, Fig. 5 &Fig. 6) between *N. stellate* and *N. rubra* (57.15%) followed by *N. stellate* and *N. nouchali* (37.5%), *N. rubra and N. nouchali* (25%). Near about 17% in between *N. rubra* and *N. pubescens, N. pubescens* and *N. nouchali, Nymphaea stellate* and *N. pubescens*(14.28%).

CONCLUSION

After analysis the selected taxa if we comprising the data of pollen morphology and free amino acids of pollen there are so many information about the relationship among the species. On the taxonomy basis *N. stellate* and *N. rubra* are less different in micro-morphological characters (Table V). Pairing affinity on the basis of free amino acids composition (57.15% between *N. rubra and N. stellata*) and pollen morphology reveals that phylogenetically two species are more or less same. So we concluded this species also phylogenetically related with *N. stellate* and *N.* rubra. But if we compared these species with *N. pubescens* few difference in pollen characters and far differences in free amino acids composition (pairing affinity of *N. pubescens* more or less 17% with rest investigated taxa). Flower morphology of *N. pubescens* flower diameter, number of petals, number of stamens, ovary diameter, number of ovary chambers and length of stigma appendages which is morphologically more related with *N. nouchali* but far difference in free amino acids composition.

The present investigation serves to elucidate exine morphology, ultrastructure, flower morphology, free amino acids of pollen and pollen production from the genus *Nymphaea* belonging to the family Nymphaeaceae and, we believe, will provide a better level of resolution regarding phylogenetic analysis of the group. Moreover, our study augments the growing number of examples demonstrating correlations among prominent ecological processes such as pollination pollen production, pollen morphology and free amino acids composition, and underscores the importance of evaluating the pollen in study of this type.

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REFERENCES

Acharya, P.J. (1980) Skin test response to some inhalant allergens in patients of naso-bronchial allergy from Andhra Pradesh. *Asp. Allerg. Appl. Immuno:* **l13**, 14–18.

Blackmore, S., Persson, V. (1996) Palynology and systematics of the Crepidinae (Compositae: Lactuceae). In D. J. N. Hind & H. J. Beentje (Eds), *Compositae*: *Systematics. Proc. Int. Conf. London (1994,* Kew: R. Bot. Gard.), pp. 111–122.

Chanda, S., Gosh, K. (1979) Apertural evolutiuon in the pollen of monocotyledons. In: *Proceedings Fourth Int. Palynol Conf. Lucknow,* **1,** 551-567.

Murthy, G.V.S. (2000) Pollen morphology of Nymphaeaceae (S.L.), *Bot. Surv. India,* **42** (14), 73-80.

Clarke, G.C.S., Jones, M.R. (1981) Cabombaceae. Review of Palaeobotany & Palynology, **33,** 51-55.

Dubyna, D.V. (1982) Nymphaeaceae of the Ukraine. Naukova Dumka, Kiev, in Russian.

Erdtman, G. (1952) *Pollen Morphology and Plant Taxonomy of Angiosperms*.Chronica Botanica, Waltham, USA.

Erdtman, G. (1960) The acetolysis method – a revised description. *Svensk. Botanisk. Tidskrift*. **54**, 561–564.

Erdtman, H. (1963) In Chemical Plant Taxonomy. Academic Press, London, pp. 89–125.

FaegriK, Iversen J. (1975) *Textbook of Pollen Analysis.* Blackwell Scientific Publications, Oxford.

Heslop-Harrisson, J. (1955) Biological flora of British Isles. *Nymphaea* L. em. Sm (nom. Conserve) *J. Ecol.,* **43,** 719-734.

Hossain, A.B.M., Islam, M.S., Khan, S. A. (2000) Taxonomy of the genus *Nymphaea* (Tourn.) L. Nymphaeaceae from Bangladesh in view of anatomical studies. *Bangladesh J. Bot.,* **30(1)**, 9-16.

Jones, M.R., Clarke, G.C.S. (1981) Nymphaeaceae- The NEP Flora. *Rev Palaeobot Palynol.,* **33**, 57-67.

Kupriyanova, L.A. (1976) Pollen morphology of Nymphaea species in the European part of the USSR. *Bot Zhurn,* **61**, 1558-1563.

Komarov, V.L. (1970) *Nymphaea* L. In Flora of the USSR. *Israel Program for Scientific Translators*.**7**, 8-12.

Mondal, A. K., Mondal (Parui), S., Mandal, S. (2009)The free amino acids of pollen of someangiospermic taxa as taxonomic markers for phylogenetic interrelationships. *Current Science,* **96(8)**, 1071-1081.

Mitra, R.L., Sharma, B. D. (1993) *Flora of India* Volume-1 Ranunculaceae-Berclayaceae.Botanical Survey of India, Kolkata. **1**, 430-431.

Miflin, B.J., Lea, P.J. (1977) Amino acid metabolism. *Annu Rev Plant Physio.***l28,** 299-329.

Mondal, A.K., Mandal, S. (1997) Biochemical and clinical studies of *Ailanthus excels* Roxb. Pollen with reference to its dispersal. *Bangladesh J. Bot.***26,** 115-120.

Molina, R.T., Rodriguez, A.N., Palacios, I.S., Lopez, F.G. (1996) Pollen production in anemophilous trees. *Grana*. **35,** 38-46.

Mandal, S., Chanda, S. (1981) Aeroallergens of West Bengal in the context of environmental pollution and respiratory allergy. *Biol. Mem.,* **6,**1-61.

Mukherjee, K.K., Chanda, S. (1990) Application of pollen phenolics in understanding plant affinity and phylogeny - a preliminary report. *Grana.***29**, 157-159.

Mitra, R.L. (1999) Nymphaeaceae. *In*: Flora of India. VolI. Bot. Surv. Ind., Calcutta.

Mondal, A .K., Parui, S., Mandal, S. (1998) Biochemical analysis of four species of *Cassia* L. *pollen. Aerobiology,* **14,** 45–50.

Meeuse, B. J. D., Schneider, E. L. (1980) *Nymphaea* revisited: a preliminary communication. *Israel. J. Bot.,* **28**,65-79.

Mondal, A. K., Parui, S. & Mandal, S. (1998) Analysis of the free amino acid content in pollen of Asteraceae species of known allergenic activity. *Ann. Agric. Environ. Med*., **5**, 17–20.

Mondal, A. K., Parui, S., Mandal, S. (1997) Aerobiology and some chemical parameters of the pollen of a few members of Mimosaceae in Burdwan District, *West Bengal. Res. J. Chem, Environ.,***1**: 12–19.

Muntendam, J.B. (1996) Morphometric patterns in the *Nymphaea alba*. candida complex. *Acta .Bot. Neerl.,***45,** 279- 302.

Nair, P.K.K. & Rastogi, K. (1963) Pollen production in some allergenic plants. *Current Science.***32**, 566-567.

Poddubnaya-Arnol'di, V.A. (1976) Cytoembryology of theangiosperms. Nauka, Moscow, Russian.

Pfahler, P.F., Linskens, H.F. (1970) Biochemical composition of maize pollen – I. Effects of endosperm mutants, waxy (Wx), shrunken (Sh.) and sugary (Su) on amino acid content and fatty acid distribution. *Theor. Appl. Genet.,* **40**, 6–10.

Punt, W., Hoen, P.P., Blackmore, S., Nilsson, S., Lee, Thomas, A., (2007) Glossary of pollen and spore terminology. Review of Palaeobotany and Palynology. **143**, 1–81.

Papchenkov, V.G. (2003) On the determination of difficult groups of aquatic plants and their hybrids. In: Papchenkov *et al*. (eds), Aquatic botany: methodology, methods: materials of the school of aquatic botany. OAO 'Rybinskijdompechati, Rybinsk, pp 82-91, *Russia.*

Parui, S., Mandal, S. (1998) Biochemistry and skin sensitivity test of the allergenic pollen of *Datura metel* L. Current Science, 74:66-68

Parui, S, Mondal, A.K., Mandal, S. (1997) Protein and free amino acid composition of the pollen of four species of *Solanaceae*. *J. Nat. Bot. Soc.*

Prance, G.T., Anderson, A. B. (1976) Studies of the floral biology of neotropical Nymphaeaceae. *Acta Amazonica*. **6**(a), 163-170

Romero, Lopes , C., De, Lucca, E.J., De, Andrade, A.M., Jacoia, Faulin, CM (1979) A phylogenetic interpretation of chromosomal and electrophoretic data in Columbiformes. *Cytologia.*44, 39– 47.

Singh, C.B., Motial, V.S., Nair, P.K.K. (1969) Pollen morphology of *Nymphaea, Plant Science*. **1,** 53-56.

Shivanna, K. R., Johri. B.M., Sastri, D.C. (1979) Advances in Pollen Spore Research, Development and Physiology of Angiosperm Pollen. Today & Tomorrow's Printers and Publishers, New Delhi.

Sadasivam, S., Manickam, A. (1992) Biochemical Methods For Agricultural Sciences.Wiley Eastern Ltd. and Tamil Nadu Agricultural University. Coimbatore.

Singh, A.B., Malik, P., Parkash, D., Gangal, S.V. (1993) Identification of specific IgE binding proteins in Castor bean (*Ricinuscommunis*) pollen obtained from different source materials. *Grana,* **31,** 376-380.

Stanley, R. G., Linskens, H. F. (1974) *Pollen – Biology Biochemistry Management*, Springer Verlag. Berlin

Sadasivam, S., Manickam, A. (1999) *Biochemical Methods*, New Age International (P) Limited Publ. 2nd ed.), Coimbatore and Tamil Nadu Agricultural University.

Sokal, R. R., Sneath, P. H. A. (1963) Principles of Numerical Taxonomy, Freeman. San Francisco. **42**, 359.
Uotila, P. (2001) *Nymphaea* L. *In: Jonsell B (ed.*), Flora Nordica. Bergius Foundation. Stockholm. **2**, 216-221.

Vance, N.C., Zaerr, J. B. (1990) Analysis by high performance liquid chromatography of free amino acids extracted from needles of drought stressed and shaded Pinus ponderosa seedlings. *Physiol Plant,* **79,** 23-30.

Wodehouse, R. P. (1935) *Pollen grains*: *Their structure, identification and significance in science and medicine*. New York: *McGraw-Hills.*

Fig. 1. Pollen production of the selected species.
Fig. 2. Mature pollen of *Nymphaea* (light microscopy). A, B. *N. stellata*. A. Polar view; B. Equatorial view. C, D. *N. nouchali*: C. Polar view; D. Equatorial view. E, F. *N. pubescens*: E. Polar view; F. Equatorial view. G, H. *N. rubra*: G. Polar view, H. Equatorial view. (Scale bar — 10µm.)
Fig. 3. Mature pollen of *Nymphaea* (scanning electron microscopy). I, J. *N. pubescens*: I. Polar view; J. Equatorial view. K, L. *N. nouchali*: K. Polar view; L. Equatorial view. M, N. *N. stellata*: M. Polar view; N. Equatorial view. O, P. *N. rubra*: O. Polar view, P. Equatorial view. Q-T. Aperture and exine ornamentation details. Q. *N. pubescens*; R. *N. nouchali*; S. *N. stellate*; T. *N. rubra*. Scale bar — 20µm (K, L, O, P), 10µm (I, J, M, N), 5µm (Q, R, S), 2µm (T).
Fig. 4. Showing different free amino acids composition of pollen of *Nymphaea* by thin layer chromatography.
Fig. 5 Dendrogram representing average linkage relationship among the species *Nymphaea*as revealed by the free amino acid composition of pollen.
Fig. 6. Pairing affinity (%) of the selected species of *Namphaea* based on free amino acids of pollen.