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RESEARCH ARTICLE

THE FREE AMINO ACIDS (FAA) COMPOSITION OF SEEDS AMONG SOME MEMBERS OF *BLYXA* THOU. (HYDROCHARITACEAE): A SYSTEMATIC APPROACH

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ABSTRACT

The seed storage free amino acids of 4 taxa of *Blyxa* were analyzed from the fully aquatic family Hydrocharitaceae by Thin Layer Chromatography. Total 22 amino acids were found, among these 3 were not identified. Considerable amount of homology were observed in between *Blyxa aubertii* var. *aubertii* and *B. aubertii* var. *echinosperma* (60%). Also another homology observed in between *B. octandra* and *B. quandricostata* (41.17%), but *B. japonica* was far differ from others analyzed taxa. The major amino acids were Amino-*n*-butyric acid, Glutamic acid, Phenylalanine, Glycine, Tryptophan, Methionine, Isoleucine and Serine. The present observation reveals that there was a correlation in between morphology and free amino acids composition of *Blyxa* analyzed taxa. Free amino acids can use as a taxonomic markers to draw the similarity among the plant taxa.

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INTRODUCTION

Hydrophytes are plants growing in water or soil covered with water. Plants of lakes, pond, streams and other aquatic environment as well as those of swamps and marshy places belong to this category. Hydrocharitaceae is a fully aquatic monocot family. *Blyxa* is one of the dominant genus under this family. Total 9 species were documented (Cook, 1996) in the world respect. *Blyxa* is distributed in warmer regions of the Old World and is naturalized in North America and Europe. Cook and Lüönd (1983) enumerated 11 taxa (nine species with two varieties) in their monograph. Kaul (1968, 1970), Ancibor (1979) and Tomlinson (1982), as well as Cook and Lüönd (1983), gave detailed descriptions of the anatomy and morphology of the genus. However, few studies have been done on their ecology except for some observations of floral biology by Cook *et al*. (1981) and Cook and Lüönd (1983). Most systematics agrees that data concerning the macro and microstructure of seeds are very significant for the classification of angiosperm taxa. Seed morphology has provided many diagnostic taxonomic characters. Micromorphology and ultra-structural data have contributed useful information for evolution and classification of seed plants and play an important role in the modern synthetic systems of angiosperms (Dahlgren, 1979, 1980), but Seed phytochemicals such as different enzymes and other organic chemicals, their structural and functional site also help the modern scientist for proper plant classification and identification in plant Taxonomy. Seeds for a particular species were collected from several individual plants of the same species to obtain a constant chemical composition, as seed 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 seeds and rapidly drying over silica gel at 30^0C, 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 seeds matures, analysis has revealed the presence of all the essential amino acids in seeds. We consider total 3 species and 1 variety of *Blyxa* for free amino acids analysis. For plant identification and correlation among species we were using amino acids as a taxonomic marker. This article will focus on the total free amino acid content and free amino acid composition of the seed of 4 aquatic angiospermic taxa belonging to Hydrocharitaceae family to assessment of homologies in the amino acid composition, we have tried to resolve the relationships among the taxa belonging to the same genus and also the phylogenetic interrelationships among the common taxa in Eastern India.

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MATERIALS AND METHODS

Field survey

Seeds were collected from mature fruits of 4 aquatic plant taxa in the genus *Blyxa* from different sweet water aquatic zone of West Bengal, Orissa and Jharkhand states of Eastern India. Herbarium specimen of the species was then prepared.

Next the plant is identified and deposited in the herbaria of Vidyasagar University. The field study was conducted in irrigation ponds for *Blyxa* species *Blyxa* populations were spread in a relatively in shallow area. (Fig 1, Table 1)

Fig. 1A

Fig. 1B

Fig. 1D

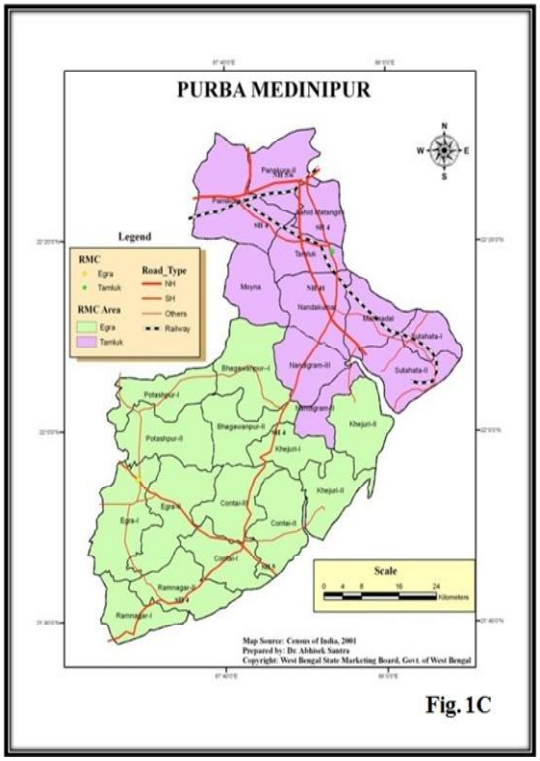

Fig. 1C

Fig. 1E

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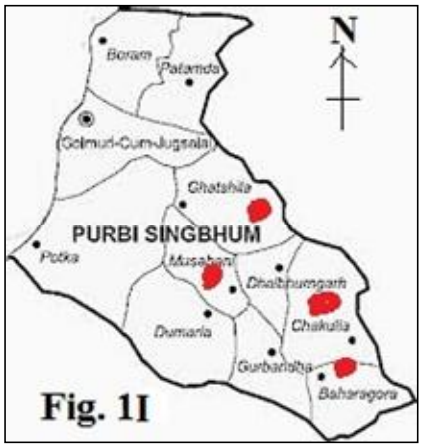

Fig. 1. Map showing the different field area of West Bengal, Jharkhand and Odisha in India (1A: India with selected sites, 1B: West Bengal with selected districts, 1C: Paschim Medinipur district with collection site, 1D: Purba Medinipur district with collection site, 1E: Howrah district with collection site, 1F: Odisha with selected district, 1G: Mourbhange district with collection site, 1H: Jharkhand with selected district,: PurbaSingbhum)

Table 1. Different field area of West Bengal, Jharkhand and Odisha

Sl. No.	Name of *Blyxa* species	Collection site Latitude and Longitude		
1.	*Blyxa aubertii* var. *aubertii*	**WEST BENGAL**		
		Dantan	21.911 ^{0}N	87.270 ^{0}E
		Keshiary	22.133333^{0}N	87.233333^{0}E
		Narayanghar	22.1514 ^{0}N	87.3929 ^{0}E
		Sabang	22.176 ^{0}N	87.601 ^{0}E
		Salboni	22^{0}38′23′′N	87^{0}20′09′E
		Keshpur	22.554497 ^{0}N	87.461149 ^{0}E
		Gorbeta	22^{0}52′N	87^{0}22′E
		Binpur	22^{0}35′00′′N	86^{0}54′55′E
		Jhargram	22.45^{0}N	86.98^{0}E
		Gopiballavpur	22^{0}13′N	86^{0}54′E
		Kharagpur	22.330239^{0}N	87.323653^{0}E
		Midnapore	22^{0}15′N	87^{0}39′E
		Ghatal	22.67^{0}N	87.72^{0}E
		Panskura	22^{0}25′N	87^{0}44′E
		Kanti	22^{0}47′N	87^{0}45′E
		Ulberia	22^{0}28′00′′N	88^{0}07′00′′E
		Howrah	22^{0}36′36′′N	88^{0}20′42′′E
		Bagnan	22^{0}28′00′′N	87^{0}58′ 00′′E

Continue……

2.	***B. aubertii* var. *echinosperma***	**JHARKHAND**		
		Ghatshila	22^{0}36′N	86^{0}29′E
		Musabani	22^{0}52′N	86^{0}45′E
		Brharagora	22^{0}28′N	86^{0}72′E
		Chakulia	22^{0}48′N	86^{0}72′E
		ODISHA		
		Baripada	21.94^{0}N	86.72 ^{0} E
		Betnoti	21.7335503 ^{0} N	86.849928 ^{0} E
		Udala	21.57 ^{0} N	86.57 ^{0} E
		Bangriposi	22.10 ^{0} N	86.32 ^{0} E
		Simlipal	21.45 ^{0} N	86.20 ^{0} E
		WEST BENGAL		
		Kharagpur	22.330239^{0}N	87.323653^{0}E
		Gopiballavpur	22^{0}13′N	86^{0}54′E
		Ghatal	22.67^{0}N	87.72^{0}E
		Bagnan	22^{0}28′00″N	87^{0}58′ 00″E
3.	***B. octandra***	**WEST BENGAL**		
		Kharagpur	22.330239^{0}N	87.323653^{0}E
		Jhargram	22.45^{0}N	86.98^{0}E
		Gopiballavpur	22^{0}13′N	86^{0}54′E
		Howrah	22^{0}36′36″N	88^{0}20′42″E
		Ghatal	22.67^{0}N	87.72^{0}E
4.	***B. japonica***	**ODISHA**		
		Baripada	21.94^{0}N	86.72 ^{0} E
		Simlipal	21.45 ^{0} N	86.20 ^{0} E
		WEST BENGAL		
		Ghatal	22.67^{0}N	87.72^{0}E
		Gopiballavpur	22^{0}13′N	86^{0}54′E
		Kharagpur	22.330239^{0}N	87.323653^{0}E

Amino acids analysis

The seeds were collected separately from at least 51 different individual plants of the 4 selected taxa and qualitative analysis of free amino acids was 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 2 are based on the mean data of 10 individual plants of each species. Free amino acids were extracted from the seeds 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.

Quantitative analysis

The total free amino acid content of the seeds 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 followed the method Sadasivam and Manickam (1996).

Qualitative analysis

Qualitative analyses of the free amino acids (Table 3) of the seeds of the investigated taxa were 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 (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 *R.f.* 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.

Data analysis

After analysis of free amino acids we analyzed the data by using 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. We also use UPGMA dendrogram for showing species similarity or relationship in investigated taxa of Hydrocharitaceae on the basis of free amino acids.

$$\text{PA (pairing affinities)} = \frac{\text{Amino acid common to the species A and B}}{\text{Total amino acids in A and B}}$$

Table 2. Total free amino acid content (%) of seeds of 4 taxa (data based on ten readings for each species)

S.No.	Name of *Blyxa* species	Mean value of total free amino acids content (µmol/mg dry wt.)	Standard deviation
1.	*Blyxa aubertii* var. *aubertii*	8.10	0.061
2.	*B. aubertii* var. *echinosperma*	9.62	0.084
3.	*B. octandra*	7.86	0.052
4.	*B. japonica*	8.66	0.089

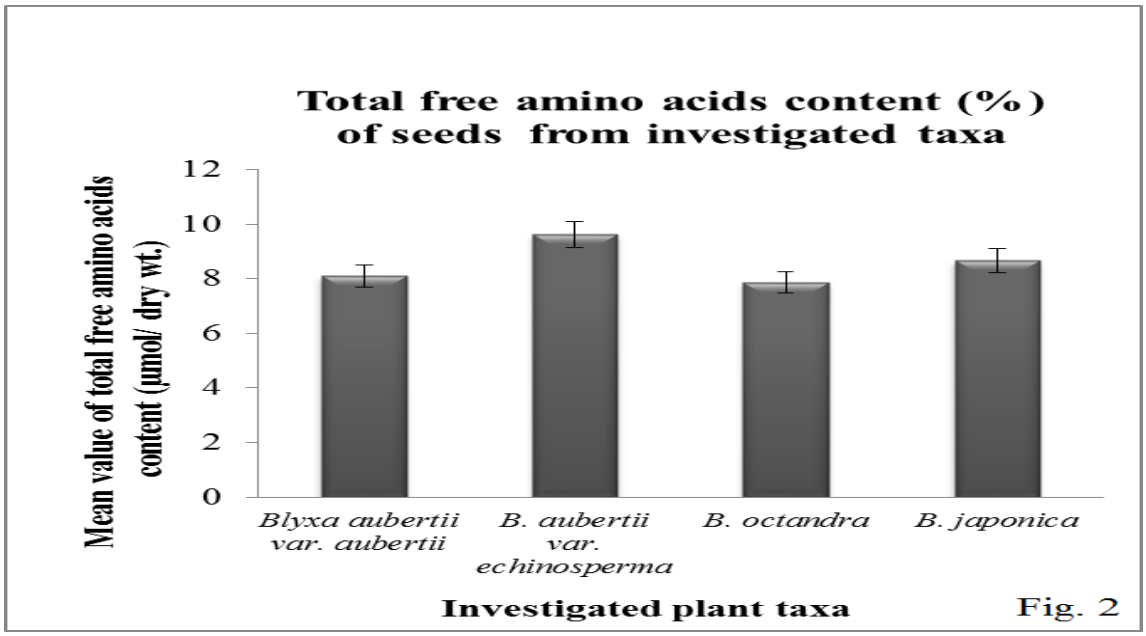

Fig. 2. The total free amino acids of investigated taxa from seed of *Blyxa*

RESULTS

Quantitative analysis

Quantitative analysis of total amino acids from seeds

Total free amino acid extract from seeds of the investigated taxa on a dry weight basis were considerably low amount (below 10%) and ranged between 7.86 to 9.62 %. *B. octandra* showed low level of amino acids (7.86%) and in *B. aubertii* var. *echinosperma* (9.62%) consisting highest level free amin oacids, followed by *B. japonica* (8.66%), *Blyxa aubertii* var. *aubertii* (8.10%). All these members showed lower than 10% but higher than 5% amino acids in seeds. (Table 2, Fig 2).

Quantitative analysis of different free amino acids from seeds

Total 22 amino acids were analyzed from seeds of 4 taxa. Arginine an important amino acids identified in adequate amount from seed of all taxa, ranged between 1.217 to 2.070%. Amino n- butyrate was present in *Blyxa aubertii* var. *aubertii* and *B. aubertii* var. *echinosperma* in large amount *B. octandra*, but absent in *B. japonica.* Phenylalanine also identified from all taxa except *B. japonica* in ranged between 1.009 to 1.641%. Leucine also present in *B. japonica* at 1.201%. Others different amino acids present in very low amount in µmol/mg dry wt. from seeds of selected taxa. (Table 3, Fig 3).

Qualitative analysis

Qualitative analysis of amino acids from seed

After qualitative analysis from seeds total 22 amino acids were found of selected taxa under the genus *Blyxa.* Out of them 3 are unknown amino acids. On the basis of our standard method total 19 known amino acids were analyzed. Maximum 14 amino acids were observed in seeds of *B. aubertii* var. *aubertii.* Total 13 amino acids were analyzed from seeds of *B. octandra* and *B. japonica.* Other was followed by 12 amino acids in *B. aubertii* var. *echinosperma*. Most common amino acids were Lysine, Alanine, Glutamic acid, Amino– n– butyrate, Dopamine, Tyrosine, Phenylalanine, Leucine, and Tryptophan found in two varieties *B. aubertii* var. *aubertii* and *B. aubertii* var. *echinosperma.* Isoleucine an important amino acid, also found in *B. aubertii* var. *echinosperma.* Another important amino acid, Valine only analyzed from seed of *B. octandra.* Simple amino acid Glycine also analyzed from seed of *B. aubertii* var. *aubertii* and *B. japonica.* Most remarkable observation was that some important amino acids such as Proline, Methionine and Aspartic acid are not present from our investigation among all selected taxa (Fig 4, Table 4).

Data analysis

The results of pairing affinities between 4 members under the genus *Blyxa* under the family Hydrocharitaceae based on the results of free amino acid analysis from seed have been summarized in Table 3.

Table 3. Comparative free amino acids composition of seeds of 4 members in *Blyxa*

Amino acid	*B. aubertii* var. *aubertii*		*B. aubertii* var. *echinosperma*		*B. octandra*		*B. japonica*	
	A	B	A	B	A	B	A	B
Alanine	+	0.042	+	0.230	-	-	-	-
Amino-*n*-butyric acid	+	1.046	+	1.258	+	0.153	-	-
Arginine	+	2.070	+	1.217	-	1.949	-	1.761
Aspartic acid	-	-	-	-	-	-	-	-
Cysteine	-	-	+	0.142	+	0.254	+	0.581
Dopamine	+	0.036	+	0.961	+	0.783	+	1.023
Glutamic acid	+	0.745	+	0.047	+	1.030	+	1.316
Glycine	+	0.446	-	-	-	-	+	0.631
Histidine	+	0.205	+	0.338	+	0.342	-	-
Hydroxyproline	-	-	-	-	-	-	+	0.069
Isoleucine	-	-	+	1.027	-	-	+	0.313
Leucine	+	0.086	-	-	+	0.458	+	1.021
Lysine	+	0.351	+	0.965	+	0.707	+	0.645
Methionine	-	-	-	-	-	-	-	-
Ornithine	-	-	-	-	-	-	+	0.432
Phenylalanine	+	1.009	+	1.641	+	1.123	-	-
Proline	-	-	-	-	-	-	-	-
Serine	-	-	-	-	+	0.548	+	0.698
Threonine	-	-	-	-	+	0.231	+	0.042
Tryptophan	+	0.954	+	1.582	-	-	-	-
Tyrosine	+	0.661	-	-	+	0.188	-	-
Valine	-	-	-	-	+	0.087	-	-
Unknown-1	+	0.098	-	-	-	-	-	-
Unknown-2	+	0.263	+	0.209	-	-	-	-
Unknown-3	-	-	-	-	-	-	+	0.220

A: Positive means present and negative means absent of amino acids, B: amount of amino acids in µmol/mg dry wt. (data based on mean value of ten reading of each taxa)

Composition of different amino acids varies greatly due to storage and handling patterns, the data were analyzed to study the evolutionary relationship as precautionary measures were taken during harvesting all seed to avoid such variation. Thus, the application of comparative free amino acids composition to determine the taxonomic groups of different taxa of the genus *Blyxa* and similarity index reveals the highest degree of pairing affinities between two varieties i.e. *B. aubertii* var. *aubertii* and *B. aubertii* var. *echinosperma* (60%) followed by *B. octandra* and *B. japonica* (41.17%), and between *B. aubertii* var. *echinosperma* and *B. octandra* (33.33%); and *Blyxa aubertii* var. *aubertii* with *B. japonica* (31.57%) and with *B. octandra* (30%). Taxa *B. aubertii* var. *echinosperma* has similarity lower than 30% with *B. japonica*. (Fig 4, Table 4)

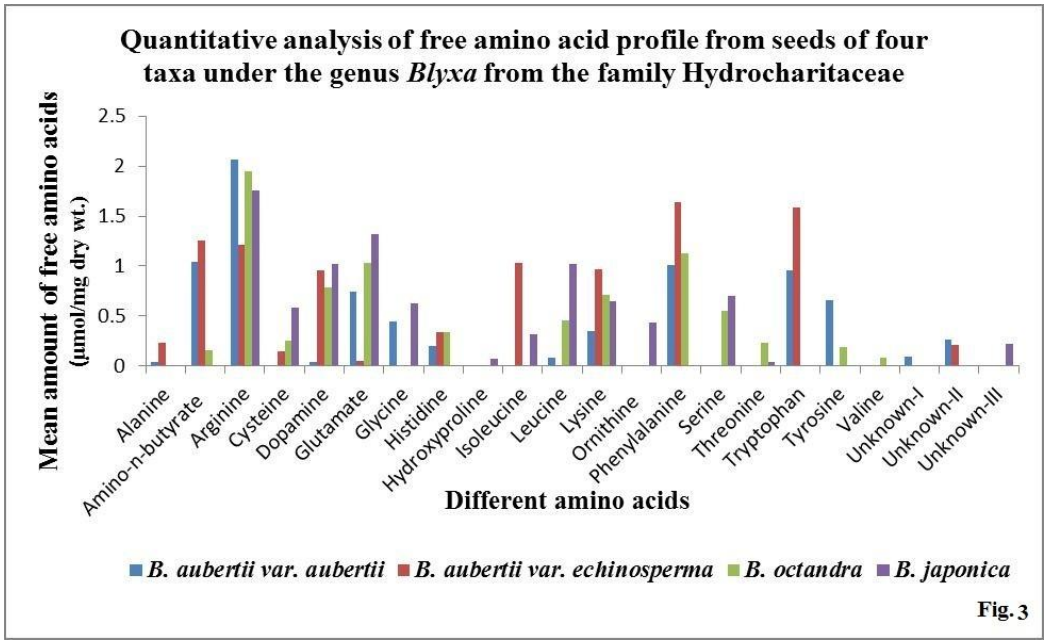

Fig. 3. Different amino acids from seeds of selected taxa

Table 4. Pairing affinity values (%) among the 4 members of *Blyxa* from Hydrocharitaceae family (based on results of free amino acids of seed)

Plant taxa	*Blyxa aubertii* var. *aubertii*	*B. aubertii* var. *echinosperma*	*B. octandra*	*B. japonica*
B. aubertii var. *aubertii*	100.000			
B. aubertii var. *echinosperma*	60.000	100.000		
B. octandra	30.000	33.330	100.000	
B. japonica	31.570	29.410	41.170	100.000

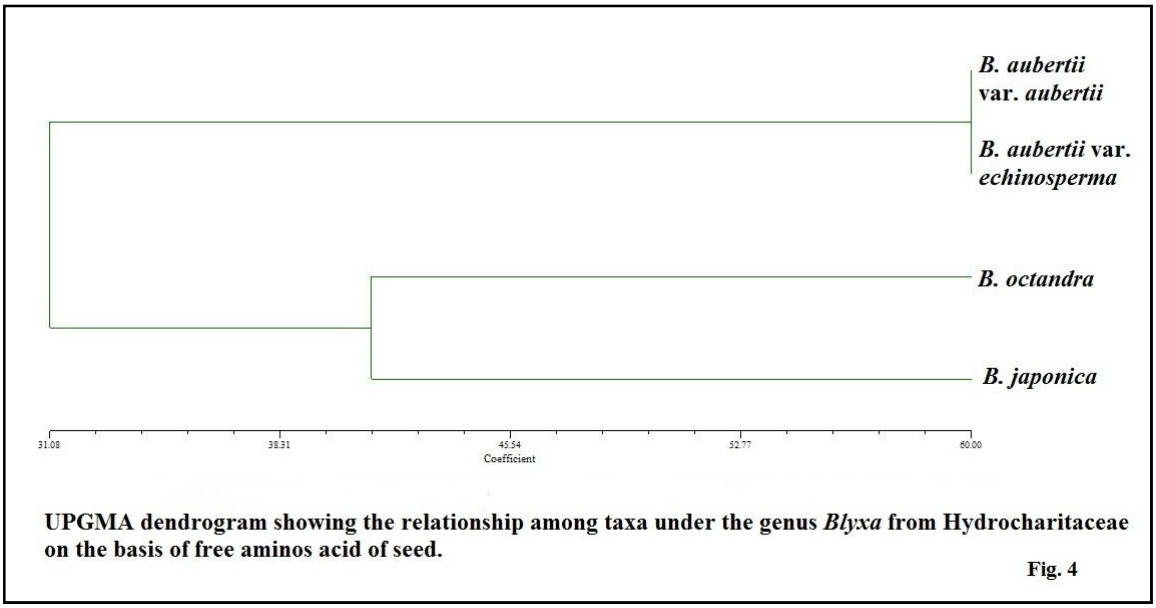

Fig. 4. The relationship among the taxa under the genus *Blyxa*based on free amino acids from seeds

DISCUSSION

After analysis of seed total 22 amino acids were observed from investigated taxa. Some are unknown but mostly common in all selected taxa. Proline was absent in all investigated taxa due to their aquatic habitat. On the other side Arginine an important amino acids also present in all the taxa which may have a role in storage and transport (Miflin, 1977). Kim *et al.* (1987) reported that the levels of Arginine, the amides (Asparagine and Glutamine) and proline increase significantly in pollen and seed under increased nitrogen fertilization. Glutamic acid on the other hand observed that in all the investigated taxa, is a common substrste of glutamine, arginine and proline, and the primary NH_4^+ acceptor as well as a product of ammonia assimilation (Miflin, 1977). So, decrease the proline accumulation level, simultaneous increase of Arginine in investigated taxa. Thus, the accumulation of proline in all the examined in seeds samples with the simultaneous absence of arginine in most all the seed could be reasoned as due to the competition 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 (Vance and Zaerr, 1990). Increased levels of amino-n-butyric acid have reflected the intensity of decarboxylation of the glutamic acid (Stanley and Linskens, 1974). Certain other amino acids were also present in investigated taxa which could not identify from standard amino acids and were categorized as the unidentified types. There may be one among the various unusual amino acids like compounds found in seed as has been reported earlier by Stanley and Linskens (1974). Threonine only present in the seed of *B. japonica* and *B. octandra* while histidine present only in seeds of *B. aubertii* var. *aubertii, B. aubertii* var. *echinosperma* and *B. octandra*. From other side in quantitative analysis of total free amino acids from seeds reveals that accumulation of free amino acids is lower than the other habitat plant groups. Arginine, Amino-*n*-butyric acid and Phenylalanine these amino acids are quantitatively high in selected taxa which may be due their aquatic habitat.

The obtained data analysis it was reveal that plant similarity on the basis of free amino acids among selected taxa, two varieties of *B. aubertii* are highest pairing affinities and followed by other two taxa *B. octandra* and *B. japonica. B. japonica* is the important species which can be isolated from other rest taxa due to its morphological variation and slightly different aquatic habitat. *Blyxa aubertii* and *B. echinosperma* look very similar in gross morphology but are distinguished by seed morphology. Cook and Lüönd (1983) considered the two taxa not worthy of the rank of species and treated them as varietal rank. This is because they found the whole range of variation in seed morphology within a single population in India. The two species are distributed from India through South-East Asia northwards to Japan and southwards to northern parts of Australia. *Blyxa aubertii* is also reported from Madagascar and Mozambique in Africa (Cook and Lüönd 1983). In Japan the distribution of both species ranges widely except for Hokkaido. Differences between the two species in habitat preference and other ecological characteristics are not known so far. Al three taxa except *B. japonica* morphologically similar in their leaf character and habitat, they grow under water in submerged condition. But *B. japonica* bottom rooted, submerged and leaf cauline i.e., terminology called vittate but others are rosulate i.e., bottom rooted, submerged and leaves in a rosette. It is the important parameter of plant classification on the morphological basis among these selected taxa. Our investigation also reveals that free amino acids composition results same to morphological plant similarity.

The result of pairing affinities between 4 taxa of the *Blyxa* in the family Hydrocharitaceae based on free amino acids analysis of seeds have been summarized on (Table 4). The amino acids composition varies greatly with storage and handling patterns, the data were analyzed to study the homology as precautionary measured were taken during harvesting all seed samples to avoid such variation.

Conclusion

Intraspecific studies become more definitive approach than the morphological studies by using biochemical characters. After analysis of seed free amino acids from 4 taxa of the genus *Blyxa* have been categorized in two clusters. UPGMA dendrogram have showing the relationship among the selected taxa. One cluster showing the two varieties of *Blyxa aubertii* are very closely related to each other like their morphological similarities. Other cluster reveals that *B. octandra and B. japonica* are closely related. *B. aubertii* var. *aubertii, B. aubertii* var. *echinosperma* are monophyletic taxa and other rest two taxa also similar another monophyletic taxon, totality i.e. called paraphyletic taxa when we consider amino acids as a taxonomic marker or a phylogenetic marker. However, it is very difficult to draw any conclusion on evolution based upon the data on free amino acid content only, as amino acid composition greatly varies with climatic and nutritional conditions as well as with storage and handling patterns. From biochemical point of view, the criteria from the seed free amino acids alone are not sufficient for correlate or delimitation among the studied taxa. In this connection more morphological and molecular studies with large number of taxa are required for sharp species correlation.

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