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Research Article

PHYLOGENETIC RELATIONSHIP OF SOME SPECIES OF *ALLIUM* L. ON THE BASIS OF MORPHOLOGICAL, BIOCHEMICAL AND CYTOLOGICAL STUDY

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ABSTRACT

Allium is the medicinally important one of the largest monocotyledonous genera. Taxonomic position of this genus remains controversial. Morphological markers alone are not adequate to achieve correct identification, interspecific relationship and proper taxonomic position of a taxon. In the present investigation, phylogenetic relationship among the four selected species of *Allium* (*A. cepa, A. sativum, A. hookeri* and *A. wallichii*) has been established through dendrogram analysis, based on some morphological, biochemical and cytological parameters. *A. cepa* and *A. hookeri* exhibited maximum and minimum bulb size and weight respectively, where as soluble root protein is higher in *A. hookeri* than rest of the three species. A comparative study based on nucleolar volume and mitotic index of *Allium* exhibited considerable variation. All the species exhibited mono and binucleolate cells. *A. hookeri* is only species which exhibits mono, di, tri and tetranucleolate cells. Largest nucleolus is observed in *A. hookeri* and smallest nucleolus is present in the cells of *A. sativum*. The *A. cepa* exhibits maximum mitotic index than the other species under study. Dendrogram analysis exhibits two hierarchical clusters- upper cluster (UC) and lower cluster (LC). *A. hookeri* is only placed in LC while the rest of the three species are placed in UC. UC has been again sub-divided into two sub clusters- UC1 and UC2. *A. cepa* and *A. wallichii* are included in UC1 while *A. sativum* is placed in UC2. Thus the present study provided useful information for the identification of the taxa, their relationship and delimitation of their taxonomic status.

INTRODUCTION

Allium L. is the largest monocotyledonous genus among the 900 world-wide distributed species (Keusgen *et al*, 2011; Govaerts *et al*, 2013; Borborah *et al*, 2014). The taxonomic position of *Allium* and related genera had been a matter of debate (Fritsch and Friesen, 2002). This genus was formally included in the family Liliaceae. Takhtajan (1997) placed the genus under the family Alliaceae, order Amaryllidales. But the Angiosperm Phylogeny Group system finally placed the genus under Amaryllidaceae family (APG, 2009). *Allium* L. has characteristic morphological features of underground storage organs comprising of bulbs and rhizomes. Majority of species of *Allium* L. are native to the northern hemisphere especially in Asia. A few species are native to Africa and Central and South America (Kamenetsky and Rabinwitch, 2006). Maximum diversity of *Allium* L. is found in North Eastern States of India, which include Assam, Meghalaya, Tripura, Manipur, Mizoram, Nagaland, Arunachal Pradesh and Sikkim. The warm tropical climate of this region provides the fruitful habitat for a wide diversity of both wild edible and cultivated species of *Allium* (Borborah *et al*, 2014).

The genus has nutritional as well as medicinal values. The onion (bulbs of *A. cepa* L.) is a popular vegetable consumed worldwide as raw and cooked forms. The garlic (*A. sativum* L.) is mainly used as a flavouring agent in food. *A. hookeri* is used as food like onion and it also used in ethnotherapy (Ayam, 2011). The young leaves of *A. wallichii* L. are cooked as a vegetable as well as the dried leaves are used as a condiment in curries and pickles. Most of the species of *Allium* have antimicrobial, anticancer, blood clotting properties, thus they are used in relieving cough, bronchitis, asthma, gastrointestinal disorders, headache and heart diseases etc. (Kumar *et al*, 2010). The chromosomes of *Allium* L. have been studied for decades for their diversity in number, size and morphology (Sharma and Aiyangar, 1961; Koul and Gohil, 1970; Konvicka and Levan, 1972; Gohil and Koul, 1980; Puizina and Papes, 1996; Fritsch, 2001). It was also reported that karyomorphologial diversity is associated with gross morphological differences in some species. The detailed comparative karyotype analysis of the

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related species has been done in many cases to describe patterns and directions of chromosomal evolution within the group and to establish the evolutionary role of karyotype changes among the species (Sharma and Sharma, 1959; Stebbins, 1971; Watanabe *et al*, 1995; Das *et al*, 1999; Vanzela, 2000; Shan *et al*, 2003). In both plant and animal cells, the nucleolus is the largest substructure of nucleus that comprises of a membrane free nuclear compartment, where the pre-ribosomal components are synthesized from several classes of ribosomal RNA (rRNAs) and different types of proteins which subsequently exported to the cytoplasm where the ribosomes are finally assembled (Carmo-Fonseca *et al*, 2000; Bersaglieri and Santoro, 2019). The nucleoli are dynamic structures showing extensive variation in size. The variation in nucleolar size is depended mainly on the activity of the organelle; fully active nucleoli are larger in size, whereas inactive nucleoli tend to remain small (Shaw and Jordan, 1995). It has been reported previously that the nucleolar size can be affected by different factors like environmental and physiological stresses (Rubbi and Milner, 2003; Olson, 2004; Boulon *et al*, 2010) and hormonal changes (Herbener and Bendayan, 1988). It has been observed that morphological and cytological parameters of nucleolus have paid negligible attention along with mitotic index and root protein content for characterization and variation study among different species of *Allium*. Thus the aim of present study is to observe the morphological, cytological and biochemical diversity of some economically as well as medicinally important species of *Allium* to determine interspecific phylogenetic relationship among the selected species of *Allium* under study.

MATERIALS AND METHODS

The selected species of *Allium* are *Allium cepa* L. (Onion), *Allium sativum* L. (Garlic), *Allium hookeri* L. (Garlic chives) and *Allium wallichii* L. (Himalayan onion), identified and collected from different regions of West Bengal. *A. hookeri* collected from North Bengal University and *A. wallichii* from foothills of Darjeeling.

The morphological, biochemical and cytological parameters were taken into account for phylogenetic analysis in the experimental plants. The bulbs were transplanted in the medicinal herbal garden of Department of Botany, Visva-Bharati in the month of November. The observations were recorded for each plant species according their day of maturation.

Morphometric characters of bulb: The height (cm), width (cm) and dry weight (g) of the bulbs of selected species are measured. At least 10 observations were made for each morphological character in two replications.

Estimation of root proteins: Roots were collected, washed under running tap water and weighed. Total root protein of each species was extracted from 0.01g of seed flour using 400µl of extraction buffer that contained 0.5M Tris-HCl, 0.01 M $MgCl_2$, 18% (w/v) sucrose and 40 mM β-mercaptoethanol having pH- 6.8. The crushed root samples were thoroughly mixed with buffer by vortexing, transferred to 1.5 ml eppendorf tubes. The extracted proteins were separated by centrifuging at 10000 rpm for 15 min and supernatant was collected and stored at 4^0C as a protein stock. The quantity of total soluble root protein was estimated by Bradford (1976) method.

Estimation of mitotic index and nucleolar volume: Nearly 2-3mm long fresh root tips were excised from the bulbs of *Allium*. The root tips of *A. cepa* were fixed in acetic acid - ethyl alcohol mixture (1:3) for overnight, followed by 5-7 minutes treatment in 45% acetic acid. Then root tips were hydrolyzed in 1 (N) HCl at 60 ^{0}C for 5 minutes and stained with 2% aceto-orcein (Sharma and Sharma, 1980). The monolayer squash were prepared on a clean grease-free slide in a drop of 45% acetic acid, sealed and observed under 400X magnification of light microscope (CH-20, Olympus) for at least 20 microscopic fields. Mitotic index (M.I.); frequency of prophase, metaphase, anaphase and telophase and nucleolar volume were calculated by the following formula:

$$\text{Mitotic index} = \frac{\text{Total number of dividing cells / microscopic field}}{\text{Total number of cells / microscopic field}} \times 100$$

$$\text{Frequency of different divisional stages} = \frac{\text{Total number of dividing cells in a particular stage}}{\text{Total number of dividing cells}} \times 100$$

$$\text{Nucleolar Volume} = \frac{4}{3}\pi r^3 \text{ (r = radius of nucleolus)}$$

Data Analysis: Based on observed variations among morphological, biochemical and cytological parameters the Hierarchical cluster analysis was performed using SPSS 16.0 (SPSS, 2004) computer software.

RESULTS

The bulbs of *A. cepa and A. wallichii* were pink colour *A. sativum* and *A. hookeri* were white colour. Significant variation was observed in the morphometric characters of bulbs among the selected species of *Allium*. Present investigation revealed that among the four selected species of *Allium*, largest bulb size and maximum bulb weight was observed in *A. cepa* and smallest bulb size and minimum bulb weight in *A. hookeri* (Table 1). A significant diversity was noticed in the amount of root protein of all the four selected species, where *A. hookeri* and *A. wallichii* contained maximum and minimum amount of soluble root protein respectively (Table 1).

Table 1 Morphological, cytological and biochemical characters of species of *Allium cepa*

Parameters	Name of the species of *Allium*			
	A. cepa	*A. sativum*	*A. hookeri*	*A. wallichii*
Bulb weight (g)	20.74	16.84	0.95	18.48
Bulb length (cm)	4.4	3.9	2.5	4.5
Bulb width (cm)	3.23	3	1.23	2.6
Mitotic index	13.88±1.36	13.22±1.42	11.68±0.70	13.27±0.81
Nucleolar volume (cu.µm)	273.422±0.84	202.807±0.92	454.896±0.79	255.243±0.59
Number of nucleolus / cell	2±0.03	3±0.01	4±0.01	2±0.05
Amount of soluble root protein (mg/g)	2.11±0.83	3±0.75	4±1.00	1.48±0.98

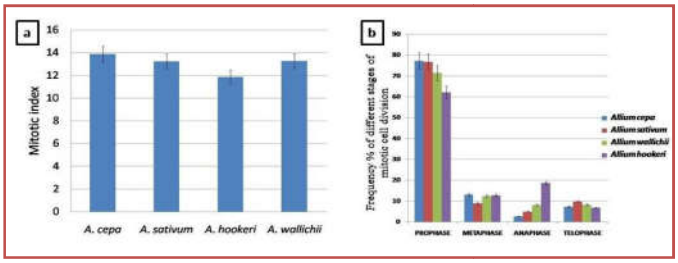

Figure 1 a) Histogram of comparative mitotic index of *Allium* sp., b) Histogram of frequency of different divisional stages among total number of dividing cells of *Allium* sp.

Mitotic index is used as an indicator of adequate cell proliferation biomarkers. The mitotic index were nearly equal in *A. cepa*, *A. wallichii* and *A. sativum* (Fig. 1 a). The metaphase and anaphase stages among four species were clearly demarcated from each other (Fig. 2). The frequency curve of divisional stages and mitotic index of each species have considerable variation among them. The frequency of prophase and telophase are nearly equal in *A. cepa* and *A. sativum* whereas lowest frequency of was recorded in *A. hookeri*. The highest frequency of anaphase was observed in *A. hookeri* and lowest in *A. cepa*. The metaphase frequency was almost equal in *A. cepa*, *A. wallichii* and *A. hookeri* (Fig. 1 b).

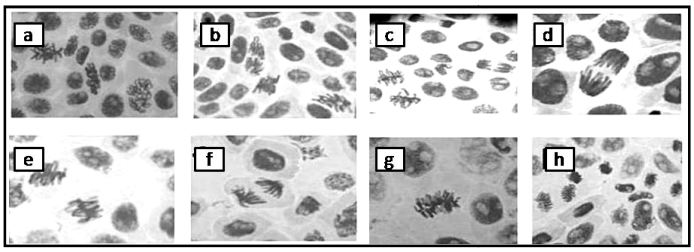

Figure 2 Different divisional stages of selected species of *Allium* (a and b) metaphase and anaphase of *A. cepa*, (c and d) metaphase and anaphase of *A. wallichii*, (e and f) metaphase and anaphase of *A. sativum* and (g and h) metaphase and anaphase of *A. hookeri*

The nucleolar morphology of all the species of *Allium* exhibited mono and binucleolate cells. Some cells with three nucleoli were present in *A. sativum* and *A. hookeri*. The tetranucleolate cells were only observed in *A. hookeri*. The comparative study based on nucleolar volume exhibited noticeable variations in compare to each other which will help in demarcation of one species of *Allium* with other. The nucleolar volume of mononucleolate and binucleolate cells was higher in *A. hookeri* and lower in *A. sativum* than rest of the two species. *A. hookeri* was the only species which exhibited all the four types of nucleolate cells (Table 2; Fig. 3). The largest nucleolus was observed in the cells of *A. hookeri* and smallest in *A. sativum* (Table 2).

Correlation analysis (Table 3) based on all concerned morphometric, cytometric and dominant biochemical features of bulb among these selected species of *Allium* indicated that all the four species exhibited significant positive correlation with each other. *A. cepa*, *A. wallichii* and *A.*

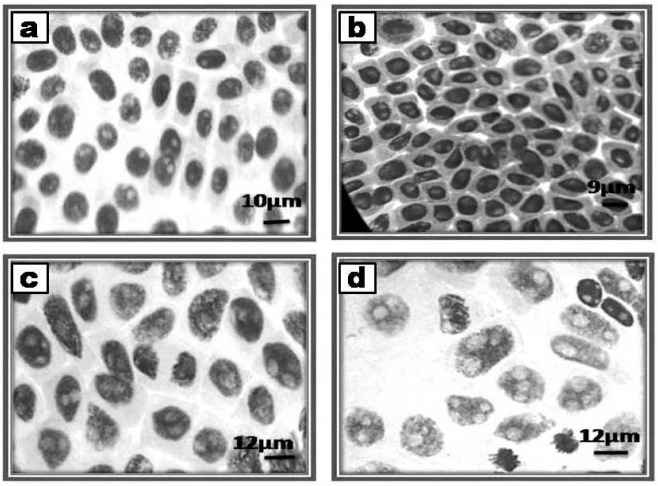

Figure 3 Nucleolar region of cells of selected species of *Allium* a) *A. cepa* , b) *A. wallichii*, c) *A. sativum* and d) *A. hookeri*

Table 3 Pearson correlation of four selected species of *Allium* on the basis of concerned morphological, biochemical and cytological characters (** Correlation is significant at the 0.01 level)

Allium sp.	*A. cepa*	*A. sativum*	*A. hookeri*	*A. wallichii*
A. cepa	1			
A. sativum	0.9998**	1		
A. hookeri	0.9975**	0.9972**	1	
A. wallichii	0.9999**	0.9999**	0.9976**	1

sativum were more closely related than *A. hookeri*. Polygraph analysis reflected the same result (Fig. 4, 5), where shape of polygraph of *A. hookeri* was quite different from other three species of *Allium* (Fig. 5). Altogether, 7 characters were taken to construct the hierarchical cluster (Table 1; Fig. 6) that exhibited inter-specific phylogenetic relationship of *Allium*. The results are shown diagrammatically in the form of dendrogram (Fig. 6). Here two major hierarchical clusters are formed among the selected species of *Allium* which are designated as - upper cluster (UC) and lower cluster (LC). *A. hookeri* was the only representative which is placed in the LC while rest of the three species are placed in UC.

Table 2 Comparative analysis of nucleolar volume of root cells of selected species of *Allium*

Name of the species	Nucleolar volume of mononucleolate cell (cu.µm)	Nucleolar volume of binucleolate cell (cu.µm)	Nucleolar volume of trinucleolate cell (cu.µm)	Nucleolar volume of tetranucleolate cell (cu.µm)	Average nucleolar volume of cells (cu.µm)
Allium cepa	220.203±0.98	313.57±0.67	0±00	0±00	273.42±0.84
Allium sativum	163.57±0.88	252.38±0.78	343.8±0.85	0±00	202.80±0.92
Allium hookeri	309.52±0.79	418.99±0.94	629.67±0.84	313.24±0.69	454.89±0.79
Allium wallichii	225.703±0.92	307.75±0.68	0±00	0±00	255.24±0.59

UC is again subdivided into two sub-clusters – UC1 and UC2. *A. cepa* and *A. wallichii* are closely associated and placed in UC1 while *A. sativum* is placed in UC2.

DISCUSSION

Previously published literature regarding phylogenetic relationship of different *Allium* secies based on morphological features (Vvdensky, 1944; Traub, 1968; Havey, 1995) and molecular analyses (Li *et al*, 2010; Son *et al*, 2010; Son *et al*, 2011; Son *et al*, 2012) are not fruitful for deciding their taxonomic ambiguity. In this work the dendrogram (Fig. 6) clearly showed that, *A. cepa* and *A. wallichii* are very closely (97%) related to each other and *A. sativum* is 93% related to *A. cepa* and 96% related to *A. wallichii* respectively. *A. hookeri* is totally unrelated with rest of these species of *Allium* on the basis of bulb morphology, mitotic index and total amount of root protein. It would be concluded from the study that *A. cepa* and *A. wallichii* are very closely related and *A. hookeri* is distantly placed whereas *A. sativum* is intermediate in hierarchical cluster position. According to

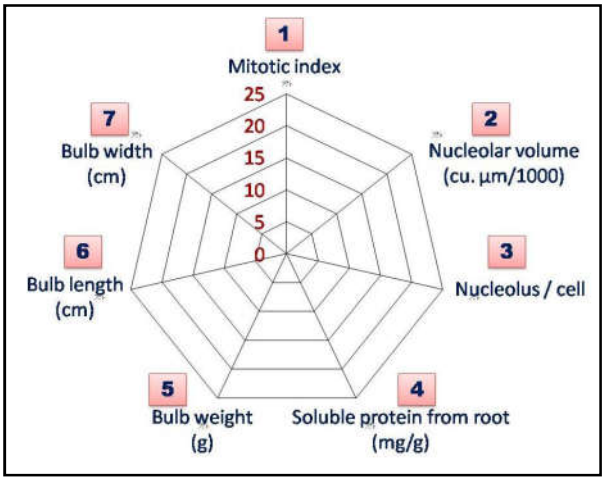

Figure 4 Showing generalized format of polygraph include concerned parameters and relative measurement scales

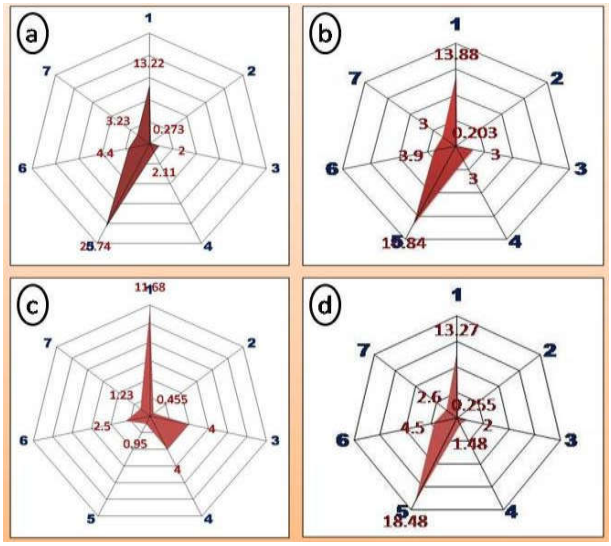

Figure 5 Polygraph showing similarities and differences among the selected species of *Allium* based on mitotic index (axis-1), nucleolar volume (axis-2), nucleolus/cell (axis-3), amount of soluble protein from root (axis-4), blub weight (axis-5), blub height (axis-6), blub width (axis-7); (a) Polygraph of *Allium cepa*, (b) Polygraph of *Allium sativum*, (c) Polygraph of *Allium hookeri*, (d) Polygraph of *Allium wallichii*

Son *et al* (2012), *A. cepa* and *A. sativum* were placed in sister clad based on ISSR banding analysis. The result of present study found in accordance with the view of Son *et al* (2012).

Mitotic index is the measure of the proportion of dividing cells in the M-phase of cell-cycle and its decrease and increase could be interpreted as cellular death and cellular growth respectively in cell proliferation kinetics (Rojas *et al*, 1993). Here, *A. cepa* exhibited higher mitotic index than other selected species, indicating that the rate of cell division is higher in *A. cepa* than those of other three

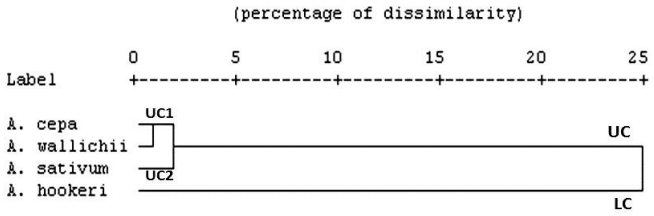

Figure 6 Dendrogram showing the phylogenetic relationship among the four selected species of *Allium* based on concerned morphological, biochemical and cytological characters together

species which is positively correlated with morphometric characters of bulb morphology. The mitotic index of *A. hookeri* is lowest which infers the rate of cell division is lower, resulting smaller size of bulb. Thus, largest size of bulb of *A. cepa* might be due to its higher value of mitotic index and smallest size of bulb of *A. hookery* could be due to its lower value of mitotic index (Table 1, Fig. 1, 2). The rate of RNA synthesis was correlated with the nucleolar volume in different phases of interphase cells of *Allium*. A rapid increase in the nucleolar volume could be noted in the early G_1 phase but the rate diminished afterwards. The nucleolar volume also correlates with the level of activity of rRNA genes (Karta *et al*, 1978; Alimann and Leblond, 1982). Therefore, nucleolar volume may be considered as an indicator of the rRNA gene activity. The larger nucleoli generally being associated with high activity of rRNA. Therefore, it can be inferred that higher RNA content in *A. hookeri* could be due to its higher nucleolar volume compared to *A. sativum*, *A. wallichii* and *A. cepa*.

The constructed polygraph based on all observations taken into account show similarity in shape, size and intersecting area of graph in *A. cepa*, *A. sativa* and *A. wallichii* in contrast to *A. hookeri*. Therefore present study is successful in providing new information about their affinities among the selected species of *Allium* which might be helpful in proper taxonomic characterization in future.

So, these parameters taken together can be used further in establishment of phylogenetic tree among different existing species of *Allium* going throughout the world. This investigation has provided new information about the taxonomical affinities among the selected species of *Allium*

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