CHAPTER 5:
CORRELATION OF MORPHOLOGICAL, CYTOLOGICAL AND MOLECULAR DATA
5. **Correlation of Morphological, Cytological and Molecular data**

An overall survey of the data accrued from the present morphological, cytological and molecular marker based studies of 27 accessions belonging to 19 species of the Indian *Allium* reveal much correspondence with the infrageneric classification proposed by Friesen *et al.* (2006). A comparison between the three data sets reveals the following interesting features.

**Subgenus Cepa**

Accessions included morphotypes of *A. cepa, A. chinense, A. fistulosum, A. schoenoprasum, A. x cornutum, A. proliferum* and *A. ascalonicum*

Analysis of morphological characters and data generated from the subsequent numerical analysis separated members of the Subgenus *Cepa* along sectional demarcations. While *A. cepa, A. proliferum, A. ascalonicum* (Subgenus/Section: *Cepa/Cepa*) and *A. x cornutum* clustered together, *A. chinense* (Subgenus/Section: *Cepa/Sacculiferum*), *A. fistulosum* (Subgenus/Section: *Cepa/Cepa*) and *A. schoenoprasum* (Subgenus/Section: *Cepa/Schoenoprasum*) were scattered under different subclusters. Though all the accessions of Subgenus *Cepa* were characterized by the presence of fistulous leaves and scapes, as well as, a well developed bulb, the above-mentioned disjunction may be attributed to presence of narrowly ovoid clustered bulbs with membranous white tunic and purplish tepals (white in case of *A. fistulosum*).

On the basis of morphological data, *Allium 1* (Chinese pyaz) separated with *A. fistulosum*, indicating their probable affinity.

Cytological study of the members of Subgenus *Cepa* show that these plants generally have long chromosomes (5.2 µm to 26.0 µm), longest ones in *A. cepa* (13.3 µm to 26 µm) and shortest ones in *A. schoenoprasum* (5.2 µm to 11.3 µm). The total somatic chromosome count varied between 2n=16 in *A. cepa, A. ascalonicum, A. fistulosum, and A. schoenoprasum*, 2n=24 in *A. x cornutum*, 2n=32 in *A. chinense*, all derived from the accepted basic chromosome number, x=8, with predominance of chromosomes with median region (m) constriction in the karyotype. Presence of two or more chromosomes with classical *Cepa* type (*A. cepa, A. ascalonicum, A. chinense*) or modified *Cepa* type (*A. fistulosum*) terminal
secondary constriction is regarded as the identifying karyomorphological character for this subgenus. Distinguishing cytological features of A. schoenoprasum were the two acrocentric chromosomes with terminal satellites on their short arms and presence of B chromosomes in 35% of all dividing cells.

The taxonomically undetermined Allium 1* showed karyomorphological similarities to the members of Subgenus Cepa, like, somatic chromosome number 2n=16, long chromosomes, maximum of median chromosomes and Cepa type secondary constriction, alluding to its affinity with them.

Previous molecular phylogenetic analyses had revealed the polyphyletic nature of members of Subgenus Cepa. In the present molecular marker study too, while arbitrary and semi-arbitrary DNA marker based analyses, combined RAPD and ISSR dendrogram also separated A. chinense, A. fistulosum and A. schoenoprasum from the core Subgenus Cepa clade, that included, A. cepa, A. proliferum, A. ascalonicum and A. x cornutum. On the other hand, the ITS sequence based dendrogram brought all these species under the same clade representing Subgenus Cepa.

Molecular marker analyses also segregated Allium 1* (Chinese pyaz) with A. fistulosum in the clade, a throwback of the result of morphological analyses.

Thus, in case of the accessions of Subgenus Cepa, results of morphological, cytological and molecular marker analyses all agree on the following observations:

i. The accessions could be separated into two broad groups:

a. a tightly associated group, that included, A. cepa, A. proliferum, A. ascalonicum and A. x cornutum, which consistently segregated as a single clade,

b. the other three, A. chinense, A. fistulosum and A. schoenoprasum, showed morphological and cytological variabilities, and as such, placements in different clusters of the dendrograms, except in the ITS sequence analyses which was effective in clustering all the accessions together
ii. The affinities of the unidentified accession *Allium*1* was alluded to by its segregation with *A. fistulosum*, in the morphological and molecular studies, although cytological analysis showed distinct differences from the *A. fistulosum* karyotype, perhaps indicative of its hybrid nature. So, while it can be said that this plant possibly belongs to Subgenus *Cepa* with close affinity to *A. fistulosum*, its possible hybrid nature has to be investigated further.

**Subgenus Polyprason**

*Accessions included A. roylei and A. carolinianum*

Position of *A. roylei* (Subgenus *Polyprason*) has always been controversial. Friesen *et al.* (2006) have supported its separation from Subgenus *Cepa*, based on morphological features, though they could not substantiate this with molecular data. The present study also revealed that while morphological characters of *A. roylei* were significantly different from other members of Subgenus *Cepa*, cytological and molecular data indicated their apparent similarity.

Morphological characters, like, presence of rhizome, membranous/papery bulb tunic, leaves with cross-sectional diameter of only 0.1-0.2 cm, hemispherical umbel, purplish to pink tepals distinguished *A. roylei* from other members of Subgenus *Cepa*, which was exemplified by its placement away from the *Cepa* clade in the morphological dendrogram, though still separating in the same clade with *A. chinense*. *A. carolinianum*, on the other hand, by the virtue of unique morphological features, like, narrowly ovoid bulb, flat obtuse apex leaf and filaments with entire bases, showed little similarity with *A. roylei*, and was segregated in a different clade.

Karyomorphological study of *A. roylei* revealed several similarities with members of Subgenus *Cepa*, like, symmetrical karyotype, basic chromosome number of 8, predominance of chromosomes with median region (m) constriction, maximum pairs of homomorphic chromosomes and two chromosomes with *Cepa* type secondary constriction. The karyomorphological analysis revealed somatic chromosome number of *A. carolinianum* to be 32 and thus basic chromosome number, x=8. Moderately symmetrical karyotypes, presence of mostly metacentric chromosomes, one group of acrocentric chromosomes, very small diffuse satellite
were other karyomorphological distinctions of this species, which were instrumental in separating these two members of Subgenus Polyprason to different clades.

The molecular marker based analysis using RAPD, ISSR and ITS markers unanimously identify A. roylei as a close relative of A. cepa, placing it with members of Subgenus Cepa in the same clade in all the dendrograms. A. carolinianum was separated from A. roylei, and placed in a different subcluster in the combined RAPD ISSR dendrogram and to different clade in the ITS sequence based dendrogram.

Concluding on the affinities between the accessions of Subgenus Polyprason, it can be said that, A. roylei is a close relative of A. cepa, and A. carolinianum is very distinct from it.

Subgenus Allium

Accessions included A. sativum, A. ampeloprasum, A. porrum and A. griffithianum

Among the accessions studied, A. sativum, A. ampeloprasum and A. porrum, all belonged to Subgenus/Section Allium/Allium, whereas A. griffithianum was a member of Subgenus/Section Allium/Avulsea.

Characteristic morphological features, like, obscure rhizome, papery bulb tunic, acute leaf apex and white tepals in flowers, resulted in the placement of the species belonging to Subgenus/Section Allium/Allium as a group, in the morphological dendrogram. A. griffithianum, however, segregated out with A. albidum, with little affinity to the other members of Subgenus Allium.

Karyomorphological study of the members of Subgenus Allium confirmed the earlier reported basic chromosome number x=8. The chromosomes were long, with predominantly median region (m) and submedian (sm) constrictions. Heteromorphic chromosome pairs were characteristic of almost all the members studied. The karyotype could be identified by the presence of typical Sativum type of secondary constriction, specific for section Allium of genus Allium (Mathew, 1996). A. griffithianum, on the other hand, in a major distinction from the other
members of Subgenus *Allium*, had modified *Cepa* type secondary constriction, and showed presence of B chromosomes.

It is known that Subgenus *Allium* is basically monoplyletic, though a few sections are deemed polyphyletic. In the molecular marker based studies, all the three datasets confirm the strong monophyly of the accessions belonging to Subgenus/Section *Allium/Allium*, when they consistently separate as a single clade. *A. griffithianum*, yet again, segregated away from the Subgenus *Allium* clade.

Thus, from the present set of analyses, it can be inferred that, Subgenus/Section *Allium/Allium* form a strongly supported clade. The identity and affinities of *A. griffithianum* was not resolved and necessitated further studies.

**Subgenus Butomissa and Subgenus Rhizirideum**

Accessions included *A. tuberosum* (Subgenus *Butomissa*) and *A. albidum* (Subgenus *Rhiziriduem*)

Prior to Friesen *et al.*’s (2006) intrageneric classification of the genus *Allium*, all the rhizomatous members were grouped under Subgenus *Rhizirideum*. They proposed segregation of Subgenus *Butomissa* from Subgenus *Rhizirideum*, but maintained that these two were closely related.

*A. tuberosum*, the only representative of Subgenus *Butomissa*, in the present study was placed under the same group in the morphological data based dendrogram along with members of Subgenus *Amerallium*, like, *A. hookeri*, *A. fasciculatum* and *A. cernuum*, owing to the presence of cylindrical bulb, flat leaves, hemispherical umbel, profusely rooting bulb, white tepals and inserted filaments. However, *A. albidum* showed low level of morphological similarity with *A. tuberosum*, and was grouped in a separate subcluster along with *A. griffithianum* (Subgenus *Allium*).

Karyomorphological studies revealed that, *A. tuberosum* is a unique autotetraploid species, with 32 chromosomes in the somatic cells, the basic chromosome number being x=8, having metacentric, submetacentric and subteloctentric types chromosomes. The karyotype was symmetrical with twelve secondary constricted chromosomes, belonging to both *Cepa* and *Sativum* types. The cytological studies
revealed similarities between *A. tuberosum* and exotic *A. albidum*, in having basic chromosome number of 8, predominance of median region chromosomes, presence of both *Cepa* and *Sativum* type secondary constrictions and symmetrical karyotype, although they differed in their total somatic chromosome counts (2n=16 in *A. albidum* and 2n=32 in *A. tuberosum*). Interestingly, karyomorphological characters of *A. tuberosum* were quite different from that of the accessions of Subgenus *Amerallium* in terms of the basic chromosome numbers and secondary constriction types.

Both the RAPD-ISSR based combined dendrogram, as well as, the ITS sequence based dendrogram, *A. tuberosum* separated out with *A. albidum*, confirming their strong affinity.

Thus, in this case the strong affinity between *A. tuberosum* and *A. albidum*, though not reflected in the morphological analyses, were confirmed by the cytological and molecular marker based analyses.

**Subgenus Amerallium**

*Accessions included A. hookeri, A. fasciculatum, A. cernuum and A. macranthum*

All the accessions of the Subgenus *Amerallium*, namely, *A. hookeri*, *A. fasciculatum*, *A. cernuum* and *A. macranthum*, segregate in the same subcluster due to presence of shared characters, like, absence of rhizome, presence of cylindrical bulb and profusely growing thick roots. Interestingly, the taxonomically unidentified species *A. clarkei* was also placed within this subcluster.

Karyomorphological studies performed also revealed gross similarities among these species, although they have variable basic chromosome numbers, i.e., x=7 (*A. macranthum, A. cernuum*), 10 (*A. fasciculatum*) and 11 (*A. hookeri*), supporting previous reports (Hanelt *et al.*, 1992; Friesen *et al.*, 2006; Wheeler *et al.*, 2013). Their symmetrical/ moderately symmetrical karyotypes showed predominance of submedian (sm) and subterminal (st) or terminal (t) chromosomes. Secondary constriction when present was of *Cepa* type. The total chromosome count of *A. clarkei* was 2n=18, and there was overall karyotypic similarity with the members of Subgenus *Amerallium*. 
All the three molecular marker based data analyses separated *A. cernuum* from the other three species. This can be justified by the presence of many “alliances” among the members of Subgenus *Amerallium*, which has been previously reported by different authors. The taxonomically contentious *A. clarkei*, which allied with members of Subgenus *Amerallium* in the combined RAPD ISSR tree was separated in the ITS data based tree.

In conclusion, it can be said that, members of Subgenus *Amerallium* show strong morphological similarities. However, the diversity of the members is evident from the cytological and molecular marker based analyses.

**Affinities of *A. auriculatum***

There was no mention about the position of *A. auriculatum* in the classification of Fresien *et al.* (2006), though according to CIMMYT-Maize Germplasm Bank (USDA/ARS), this species is a native of the Indian subcontinent and member of Subgenus *Cyathophora*. Subgenus *Cyathophora* of Fresien *et al.* (2006) was a small group of only 4-5 species, distributed in the Himalayas, which they carved out from *Rhizirideum*. There were no other resources available regarding the affinities of *A. auriculatum*. Huang *et al.* (2014) from their studies concluded that the Subgenus *Cyathophora* as proposed by Fresien *et al.* (2006) was not monophyletic, and reassigned the species into subgenera *Amerallium*, *Butomissa*, *Rhizirideum* (s. str.) and *Cepa*.

In the present analysis of morphological data, *A. auriculatum* separated along with *A. schoenoprasum* (Subgenus *Cepa*), due to the presence of shared characters like linear leaves with circular cross-section, purple coloured tepals and inserted filaments. Both these species segregated together in the combined RAPD ISSR marker based dendrogram, but analysis of the ITS sequence data separated *A. auriculatum* from members of the Subgenus *Cepa* and formed a clade single. Still this dendrogram clustered that members of subgenus *Cepa* and subgenus *Polyprason*, closer to *A. auriculatum* than all other studied subgenera.

So, from this study it can be concluded that, in most cases, morphological, cytological and molecular data sets show strong correspondence, though they do disagree in some cases.
The phylogenetic framework of the genus *Allium* generated in the present study was in agreement with the one proposed by Friesen *et al.* (2006). Since the sampling was not extensive, and restricted predominantly to the Indian *Allium* species from Eastern India, there were a few inconsistencies. Members of the six subgenera studied, segregated as separate clades with high levels of BS support. There are four basic chromosome numbers, i.e. 7, 8, 9 and 10, in the genus. 90% of the Old World species have $x=8$, a few $x=7$ and 9, and $x=10$ is restricted only to a few species of Russian origin. 90% of the North American species, on the other hand, have $x=7$, as the basic chromosome number. Regarding the ancestral basic chromosome number, it has been hypothesized that in the section *Molium*, a group of Mediterranean species, the basic numbers 8 and 9 have arisen from seven in the form of an ascending series, i.e. $7\rightarrow8\rightarrow9$, evidence in the section *Codonoprasum*, which includes European and West Asiatic species, suggests that the basic number eight has given rise to both seven and nine. All these lineages can be identified as separate branches of the phylogenetic tree (Fig 52). Superimposition of the haploid karyotype on the phylogenetic framework reveals interesting correspondence of the molecular and cytological studies, and reaffirms the previous and present studies.
FIGURE 52: Mean haploid idiograms superimposed onto a phylogenetic framework of genus Allium. Basic chromosome numbers (x) are given above respective clades. Strength of branch (*** =supported by three molecular markers, *=supported by one molecular marker). Subgenera of genus Allium are indicated on the right of the figure. Alphabet at the top of each idiogram corresponds to the name of the species. (bar=5µm)