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Methodology for Collecting and Preparing Herbarium Specimen of *Allium*

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Taxa belonging to the genus *Allium* are bulbous or rhizomatous with hollow or flat leaves and small delicate flowers, therefore preparing an ideal herbarium specimen is difficult. Quite often some of the characters are not well preserved while processing for herbarium specimen; occasionally they are misunderstood for characters therefore defeat the very purpose of their use as source for taxonomic study. In this paper the authors have made an attempt to provide methodology on collection and preparation of herbarium specimens of different species of *Allium*. Information provided herein is primarily based on the authors' field experience and experimental and herbarium study on the genus in the Indian context. Most significant observations to be recorded in field during collecting include characters of root and bulb/rhizome, leaf, scape and flower. The paper also includes modified procedures for 'difficult to preserve herbarium specimens' with special notes on plant odour, flower colour and leaf colour. This can be broadly applied to taxa belonging to other bulbous groups with appropriate modification. Illustrations provided in this paper depict the representative types to facilitate better understanding by the readers.

Allium is among the largest monocotyledonous genera comprising of more than 800 species (Fritsch *et al*., 2010; Fritsch and Abbasi, 2013). Many economically important crops such as garlic, onion, leek, shallot, bunching onion, chives and Chinese chives, giant onion, and ball-head onion are cultivated as vegetables, spices, condiments and ornamentals (Fritsch and Friesen, 2002). The genus *Allium* shows remarkable variation in vegetative characters [underground storage organs–bulbs, rhizomes, swollen/ fleshy roots; leaf-size, shape, anatomy (section), sheath/ lamina ratio], mode of propagation (seed, bulb, rhizome, bulbils), floral characters (morphology of spathe, pattern of opening of flowers, tepal size and shape, stamen length and colour) and seed characters (size, shape, and ultra-structure of seed coat). Variation also occurs in growth form (annual to perennial habit) and winter dormancy, biochemical composition, chromosomes and ploidy levels.

In India the genus is represented by about 35-40 species that occur in temperate and alpine region of Himalaya (Polunin and Stainton, 1984; Pandey *et al*., 2008) including major cultivated species such as onion and garlic. Alpine-subtemperate region (2500-4500m) of the western Himalaya with about 25 taxa is the zone of rich species diversity (Gohil, 1992; Pandey *et al*., 2008). Assessment of species distribution *vis-a-vis* germplasm collected and conserved under genetic resource programme has shown thrust in this direction (Negi and Pant, 1992; Pandey *et al*., 2005b; Pandey *et al*., 2008; Khosa *et al*., 2014; Pandey *et al*., 2014). Hitherto unexplored areas are the potential regions for new variability as well as of probable new taxa.

Some of the important characteristics for identification of different species of *Allium* are bulb or rhizome (shape, outer tunic/membrane), leaf (succulent, flat, fistular, keeled), and inflorescence (inflorescence shape, flower colour, perianth shape and length) and presence of bulbils (Fig. 1; Appendix 1). Additionally some ultra-structures of stamen (teeth on inner ones) and orientation of ovarian crests are important for species identification in the genus (Wheeler *et al*., 2013).

Among vegetative characters used for taxonomic identification the bulb coat characters are of importance for delineation of taxa. Species with cellular-reticulate bulb coats, with considerable variation in the patterns of cells on the inner surface of the outer bulb coat are particularly important for identifying species. Outer bulb coat with an open-celled "mesh" tunic has lesser moisture retaining properties as compared to that of the closed-cell coat that acts as effective barrier to moisture (Rola, 2014).

Well preserved and documented herbarium resources are of high value for taxonomic research globally besides their use in molecular study (Rogers and Bendich, 1985). Heavy dependency on herbarium

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material on the genus, lack of access to study material in field, and different period of availability of vegetative and reproductive stages has lead to difficulty in taxonomic and revisionary studies in this genus (Friesen *et al*., 2006).

Bulbous taxa are categorized as group "difficult to collect and process as herbarium specimen" for a plant collector (Pandey *et al*., 2013). Many characteristics of systematic value in the genus *Allium* (root morphology, bulb coat colour, leaves if hollow or flat, keeled beneath, inflorescence– globose, spherical or hemispherical, scape solid or hollow, shape and colour of perianth segment, and stamen characters) are lost while drying, hence difficult to represent in the herbarium specimen. This paper provides a comprehensive methodology to facilitate collecting and processing of herbarium specimens and recording of essential data pertaining to species delineation.

The present study is an outcome of work done under project on "Genetic Resources and Systematic study of Alliaceae in India–*Allium*" at the ICAR-National Bureau of Plant Genetic Resources (NBPGR), New Delhi. Data were collected while germplasm collection trips undertaken during 2009-14 and observations noted during experimental study carried out at NBPGR, New Delhi using standard IPGRI passport (http://www.ecpgr.cgiar.org/fileadmin/bioversity/publications/pdfs/728_Descriptors_for_*Allium*__*Allium*_spp._.pdf). Additionally, herbarium based study using 700 specimens at NBPGR herbarium (NHCP), BSI (BSD), FRI (DD) and NBRI (LWG) were used apart from online virtual herbaria (K, P, E, PE, GAT). For validation of characters selected e-resources in major herbaria (www.efloras.org/florataxon.aspx?flora_id=2&taxon_id=101086;,http://www.efloras.org/florataxon.aspx?flora_id=5&taxon_id=101086;http://www.efloras.org/florataxon.aspx?flora_id=1&taxon_id=101086, USDA Plant Database, http://linnean-online.org/view/collection/linnean=5Fherbarium/*Allium*.html) were also used. Herbarium techniques for collecting and drying were used as per the standard procedures (Jain and Rao, 1977) with modifications for processing of leaves and bulbs. Methodology was validated by the authors while collecting in fields and processing of material at the National Herbarium of Cultivated Plant (NHCP), New Delhi.

Herbarium specimen prepared of a material only at vegetative or reproductive stage is inadequate and therefore of less value for use in systematic study. For a cultivated or wild species one should take as many observations as possible at various growth stages in the field during collection and while processing and grow out in experimental study. The collector needs to go during early growth period (in December-January) for vegetative material and for flowering specimen (in May-June when bulb mature). It demands visiting the same area at least two times or else hunt for early and late sown plants. It is recommended that collected material should be pressed fresh (especially flowers) in the field or collected in polythene bag (closed with rubber band) during collection. Standard digging instrument, blotters, plant press, camera, hand lens, bottles (with 10 per cent FAA) were used following methods given by Jain and Rao (1977).

Specimens of cultivars show variation in morphology of mature bulb coat, colour, size of bulb, shape, pungency, etc. and therefore representation of distinct type is suggested. The specimen with detailed notes on specific features, hand drawn line diagrams of bulb shape on the herbarium sheet and photographs with close up of parts can be put on sheet along with magnification range. Additionally wet collections can be made to depict variation in bulb characters especially the shape, size, etc., although colour is lost during preservation. The bulb coat can be separately collected and preserved for a longer period without deterioration. Among the reproductive characters, the flower and seeds being small are difficult for study in field; perianth is fragile and loses colour and shape and study of internal structures therefore is difficult. If herbarium specimen is collected for molecular study, mounted specimen should not be treated with any chemical protectants other than deep freezing treatments; it should have label clearly demarcating the purpose of study (Wheeler *et al*., 2013).

Given below are some specific observations to be recorded while collecting in the field and preparing herbarium specimens:

Habitat

Notes on habitat conditions for different species of *Allium* (wild, semi-domesticated or cultivation), collected from farmer's field, kitchen garden or experimental conditions; record data on frequency of occurrence (abundant, frequent, rare, occasional, etc.), associated species, soil type (rocky, saline, sandy), colour and texture, topography, etc., are essentially needed for wild plants. Data on latitude, longitude and other geographic parameters can be recorded using GPS; this can help in

Fig. 1. Illustrations showing characters to be noted for different parts of *Allium:* (top row) fleshy roots; fasciculate roots; bulb with fibrous coat; (middle row) bulb with membranous coat; freshly harvested bulb with normal roots and triquetrous leaves; (bottom row) hollow leaves shown by insertion of strip on cut end; inflorescence showing exerted anthers.

locating the species at similar location elsewhere in the area of exploration.

Habit

It is often difficult to collect a complete specimen with fully mature bulb and leaves and floral parts together for preparing into a herbarium specimen; in many cases bulb may be fully mature but leaves, scape or inflorescence withered away. The freshly dug out specimen with immature bulb does not really represent the actual shape of the particular species. Fresh harvest of *Allium* sold in the market may only represent part of species (leaves of *A. tuberosum, A. hookeri*) and young bulb with leaves (*A. cepa, A. chinense, A. ampeloprasum*). In such cases one should try to represent different stages of plant at vegetative growth (bulb, leaf, etc.) and reproductive stages (scape, inflorescence, flower).

Bulb

Tunicated bulb formed from basal leaves is a well demarcating character that helps delimiting *Allium* from many bulbous taxa (*Amaryllis, Drimia* called wild onion in many regions of India). Notes/observations on bulb (single or in cluster, cylindrical, bulb shape, size, level of development, membrane character–outer and inner coat colour, texture, etc.) are important in identifying different taxa of *Allium*. Collector should ensure that intact bulb is collected, or if peeling off collect the sample of membrane in paper pouch. Notes on bulb colour at harvest/maturity are to be recorded; in an immature bulb the coat colour often an unstable character and can mislead in the identification of species. Take observations of the inner wall of outer coat making peel mount and examining it for cell shapes under 10X magnification.

Presence of underground bulbils is an additional character that delimits taxa. Bulbs in single or in cluster, size and shape of the bulb, and leaf characters can distinguish species even at vegetative stage (*A. chinense* vs. *A. cepa* var. *cepa*, *A. cepa* var. *aggregatum* and *A. proliferum*). *A. chinense* has cylindrical-long necked bulbs, triquetrous leaves and white membrane whereas in others the leaves are fistular (round/elliptical in section, bulb not cylindrical with short neck, membrane white or shades of brown, violet, lilac).

Usefulness of bulblets/bulbils to serve as taxonomic identity especially when detached from the mother plant is questionable. Bulblets (cloves) of garlic (*A. sativum*) and *A. ampeloprasum* (great headed garlic) can be distinguished on the basis of size and texture of bulb coat; the former with thin membranous flat leaves but latter has thick, leathery and keeled leaves. Underground/aerial bulbils are always thick walled, smaller than the normal bulblets. Underground bulbils are located below the bulblets or may be with stoloniferous roots. Aerial bulbils are smaller than the underground bulbils, borne in the scape/floral heads. While collecting the position, size and shape (morphological structure) of the bulbils must be carefully noted. In case of doubt grow out tests are essential.

Rhizome

Presence of a developed rhizome, its position–horizontal or vertical is characters associated with subgenus and section. Subg. *Amerallium*, rarely produce a notable rhizome; subg. *Anguinum* and *Melanocrommyum* consist of small rhizomes and no bulb whereas subg. *Butomissa, Rhizirideum, Allium, Cepa, Reticulatobulbosa* and *Polyprason* have both rhizome and bulbs (reduced, modified or condensed state). Well developed rhizome is generally deep-seated and collector should use digging tools to take out carefully. Presence of thick rhizomes in a dried specimen can only be seen if specimen is dug properly and processed (*A. tuberosum*). Condensed rhizome in garlic and onion can be visible through the vertical section of bulb. Fibrous coat on bulb/rhizome needs special observations for colour and texture.

Root

Root structure is very important character at infrageneric level identification. Fleshy root character associated with subg. *Bromatorrhiza- A. hookeri, A. fasciculatum* and *A. macranthum* is distinct feature to delineate species. Fleshy roots generally dry or shrink on processing and may be noted or sketched on herbarium label; photo of freshly dug out sample can serve the purpose.

Leaf

Number of leaves, shape (triquetrous, round/channeled, flat, keeled) can be examined in fresh material. Longitudinal/transverse section of leaf can delimit *A. cepa, A. cepa* var. *aggregatum, A. fistulosum* and *A. proliferum.* Keeled nature of leaf can distinguish *A. ampeloprasum* at pre-flowering stage from *A. sativum.* Colour of leaf, shape of the leaf tip, leaf orientation with respect to vertical axis, density of leaves, texture-membranous or thick, leaf length with respect to scape are important characters to identify taxa. The two species,

A. auriculatum (subg. *Cyathophora* sect. *Coleoblastus*) and *A. przewalskianum* (subg. *Rhizirideum*) co-occur on dry slopes, ravines and rocky crevices are difficult to distinguish in routinely preserved herbarium specimens as fistular leaf and perianth characters of the latter species, as a distinguishing trait from the former species are lost during processing.

Inflorescence

Shape of the inflorescence- globose, compact; spreading pattern, opening of flower (regular, irregular, centrifugal), number of flower opened at a time, scape length (longer or shorter than leaves), shape (cylindrical-tapering), hollow or solid (at maturity), shape of flower, can be noted at any stage of collection to drying of herbarium. In pressed condition the inflorescence of *A. cepa* var. *cepa* and *A. cepa* var. *aggregatum* are difficult to distinguish; others like *A. semenovii* and *A. macranthum* can be quickly identified with distinct inflorescence.

Flower

Macroscopic characters as colour, streak on the perianth, orientation of perianth in open flower, perianth shape, and stamen length with respect to perianth and orientation; shape of the basal part of inner filament, orientation, presence or absence of teeth and its shape can distinguish closely related taxa. *A. ramosum* and closely related *A. tuberosum* differ in flower characters, the former has pink mid line on the dorsal side of perianth. Orientation of perianth and stamen in an open flower is lost during drying so need to be recorded in field data book. Notes on flowering time (day hours) vs. stage of flower (early-late), pollinators' specificity should be noted. Among the insect pollinators *Aphis dorsata* the common honey bee, and *A. cerana* and *A. mellifera* are the most frequent visitors to *Allium cepa* and *A. schoenoprasum* whereas the common fly prefers to visit *A. ampeloprasum* (Kumar and Gupta 1993). A note on these should be recorded.

Seed

Seeds are very small and not readily observed in the field for detailed micro-characters; however an outline and overall shape (angular, round, broader) can be noted with help of hand lens. Seeds shape round (*A. victorialis*), sickle shaped (*A. carolinianum*), tear shaped (*Nothoscodum gracile*), etc. can be drawn on the field note book.

Odour

Onion or garlic type odour on crushing can be noted when sample is harvested and recorded on label. For example *A. tuberosum* and *A. schoenoprasum* have mild onion flavour whereas *A. ampeloprasum* has garlic odour.

Photographs and Other Material

Plants at different stages of growth, inflorescence at different angles, flower when fully open (close-up view), perianth and anther orientation need to be photographed. Additionally, the dried parts (flowers, stamens, leaves may be collected) and information on important features and ethnobotanical data should be recorded in herbarium specimen label.

Synopsis of Specific Notes

Field Observation

- **Habitat/ecological aspects:** record habitat details using standard IPGRI passport (http://www.ecpgr.cgiar.org/fileadmin/bioversity/publications/pdfs/728_Descriptors_for_*Allium__Allium*_spp._.pdf) with niche-specific information, distinct habitat (locality latitude, longitude using GPS), altitude, soil type, moisture, atmospheric temperature, topography (slope condition -steep/shallow; rocky, crevices, cliffs), exposure/sun exposure and if endemic. Note-sympatric allopatric species; habitat condition, co-associated species, etc.
- **Life form:** perennials/annuals, perennating organs–bulb/rhizome, leaf, scape visible at the time of visit.
- **Co-associates:** associated plants in field.
- **Texture and colour of plant parts:** flower colour (use colour chart), glaucous, texture, shininess; outer coat if membranous, coriaceous or fibrous, if fibrous loosely or finely knitted, colour of coat on harvest, roots fleshy, leaf thick, thin, erect or pendent, bulb shape, size, colour, inflorescence as seen fresh (flower opening pattern, spathe valves in fresh; compact/spreading, shape seen as 3-d in fresh but 2-d in press are lost in drying process).
- **Stage at which observations recorded (bulb and leaf):** prepare herbarium specimen of vegetative/reproductive plant over various growth stages; variation can be noted at early stage (1 month, 2 month), fully mature plant (at flowering). Plants at

seedling stage do not show leaf and bulb characters at two month old stage. They may show the hollow or flat type of leaves. Determine the standard stage for taxa at which the collection is to be done. Bulbils if present should be represented with mother plant.

- **Flower characters:** pattern of opening of flowers, perianth orientation-horizontal or erect, filaments longer/shorter than perianth, striations on the dorsal side of perianth, colour and length of stamen, etc. to be noted in fresh. Opened flower can be dried with care and mounted in cotton strip/kept in pouch. Field/hand held microscope can be used for micro characters.
- **Phenology:** early/late flowering, length of flowering time, pollinator specificity, if any.
- **Extreme variants:** variants with respect to good taxonomic characters within population (field and herbarium based study); variation range in a species with high ecological amplitude (*A. carolinianum*).
- **Illustrations:** drawings/cross sections of parts, leaves/scape (hollow or solid), sketches/line diagrams to show details.
- **Supplementary information:** photographs of field/ population; close up of the leaf, bulb, flower, anthers; etymology, local use, ethnobotanical information/ local name, etc. may help in identification.
- **Equipments:** humidity recording equipment, relative humidity meter, soil PH meter, GIS (location data recording).

Herbarium Preparation

While making herbarium specimen, one needs to depict:

- **Tall plant:** to be bent into parts/small parts: cultivated (*e.g. A. ampeloprasum, A. cepa*) groups with bigger upper, lower leaf surfaces, bending long scape; basal, middle and tip parts of leaf portion (in case of long leaf).
- **Bulb:** expose the bulb membrane showing outer and inner coat characters; bulb in full or section (poured with salt/disinfectant on the cut), vertical section to show arrangement of basal leaves. Membrane from completely dry or mature bulb can be preserved during processing of herbarium specimen (collect and dry it separately in pouch).
- **Leaf:** cross section, leaf insert (cut/oblique) with coloured stick/strip to show the hollowness; longitudinal section of leaf from bottom- tip to show the entire length wise and also same for scape. To depict the character of density in leaf, trim some leaves (only in dense condition) leaving only 5-6 cm basal part.
- **Pseudostem:** length (size); arrangement of leaves to be noted; one should try to alter orientation/ arrangement of leaf.
- **Inflorescence:** take measurements of diameter and draw line diagrams to depict the shape of the inflorescence; spathe is generally well preserved, yet splitting can be noted or drawn. Similarly the pattern of flower opening (centripetal/centrifugal vs. irregular) are sometimes hidden in pressed state so may be depicted pictorially or with photograph. Compact vs. loose arrangement of flowers in inflorescence is difficult after pressing, so data to be recorded in label or notes.
- **Flower:** colour of flower, perianth shape, perianth orientation in fully wide open flower are the characteristics for differentiation but generally lost while drying. Anthers exerted or included, stamen colour, orientation of anthers, micro-characteristics of stamen (base of filament, toothed or not), ovary shape are to be examined using the microscope. Take a flower and wide open and dry in a small piece of blotter after opening/slitting it wide to show all parts clearly. Some of the fully mature flowers, buds can be separately dried and put in the pouch for later study (detaching plant part to be studied, soak in 5-10 percent salt water in ambient temperature for 8-10 hours, spread on mounting glass and put under dissection microscope). Put dried flowers, scape, capsule in a pouch; draw diagram of individual flower.
- **Illustration:** prepare on-spot/free hand drawing to show the unique part (anther, fibrous nature of membrane; photos in close view from top of flower, longitudinal view of inflorescence to show anther viz. tepals ratio (anthers included or peeping out).
- **Treatments:** the bulb may continue to grow while in plant press; apply artificial heat, alcohol or formalin to kill the tissue (bulb/rhizome). Fleshy parts can be dried slowly through blotters/corrugated sheets. However, standard methods used for fleshy material

can be used with modification (Jain and Rao, 1977). The authors have found significant use of salt powder to facilitate fast drying and no fungal/microbial growth on tissue.

- **Extra material:** complementary parts-use paper pouch to put roots, bulbils, underground bulbils or left over parts with herbarium specimen. One flower to be split open longitudinally and pressed with corolla spread out to show androecium and gynoecium; corolla to be pressed separately in tissue paper on tepals/non- absorbent tissue paper; large parts/fleshy or those with complex flowers in wet preservation in alcohol/formaldehyde (4 per cent); apply even pressure on leaves and flowers to prevent sticking. To depict the variation in bulb characters of a cultivar one can use wet preservation method.
- Completely processed specimen can be mounted as per standard herbarium procedures (Jain and Rao, 1977). Mounting and stitching with thread vs. glue application depends on discretion of user. If the herbarium material is to be used later for some study (biosystematics, chemotaxonomy, or molecular work) one should avoid sticking with glue or any adhesive.

The NHCP has mandate to build-up herbarium collections of crops/crop cultivars and its wild relatives. Herbarium resources available with these details are of much value for preparing key to identification based on leaf, bulb and seed characters. Standardized methodology given here on the genus *Allium* may be used for collection and processing of material as herbarium useful for plant genetic resource related studies.

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Appendix 1

Significant features to be noted for infra-generic categories

S. No.	Infra-generic category (subgenus)/species	Plant parts/characters
1	**Amerallium**	
	A. hookeri, A. macranthum, A. fasciculatum	Root shape and fleshy character; bulb outer coat, leaf shape
2	**Anguinum**	
	A. victorialis, A. pratii	Rhizome shape/size, leaf shape and width, vein characters
3	**Butomissa**	
	A. tuberosum, A. oreoprasum, A. ramosum, A. gilgiticum	Rhizome, bulb shape/size, outer and inner coat colour, texture, leaf in section; flower- perianth shape, colour of the dorsal mid-line on perianth
4	***Allium***	
	A. sativum, A. ampeloprasum, A. griffithianum	Bulb shape, number, coat texture; presence of bulblets; leaf shape, density; presence of aerial bulbils
5	**Cepa**	
	A. atrosanguineum, A. semenovii; A. cepa, A. fistulosum; A .longistylum; A. schoenoprasum; A. chinense	Rhizome size; bulb shape, bulb coat characters; leaf fistular
6	**Polyprason**	
	A. stracheyi, A. roylei, A. consanguineum, A. carolinianum	Bulb shape, single/clustered, rhizome size, if produced into a neck; leaf shape, texture (leathery); tunic outer colour, texture- fibrous, coriaceous, brown
7	**Cyathophora**	
	A. auriculatum	Bulb shape, coat colour, texture; leaf shape, leaf in cross section, scape size, erectness/orientation
8	**Melanocrommyum**	
	A. atropurpureum, A. chitralicum, A. loratum	Flowers if showy, nectaries located in the lower half of the ovaries; excretion through spur or rather than short tube; excretory tubes bent downwards.
9	**Reticulatobulbosa**	
	A. humile, A. schrenkii, A. lineare, A. sikkimense, A. tenuicaule	Bulb shape, number, tunic colour, texture; leaf length, flat, spathe-valve number, perianth colour, filament length, scape length, ovary apex orientation
10	**Rhizirideum**	
	A. przewalskianum	Bulb shape, tunic character, leaf length, if channelled, tepal: filament length