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Garlic: Its Importance and Biotechnological Improvement

Abhilash Nair[1], Anil Khar[1], Amandeep Hora[2] and CP Malik[2]*

[1]Directorate of Onion and Garlic Research, Rajgurunagar, Pune, India
[2]School of Life Sciences, Jaipur National University, Rajasthan, India
Email: *cpm_malik@yahoo.com; [2]aman.hora 87@gmail.com

ABSTRACT

Garlic bulb is an important seasoning ingredient and used in countless world's cuisines. Clonal lineages within this species exhibit high degree of phenotypic diversity in bulb size and colour. Present review describes morphology, taxonomy, chemistry, cultivation, medicinal properties, post harvest processing and biotechnological approaches for improvement. Special emphasis is laid on the genetic variations of garlics with special emphasis on Indian accessions. The available results are divergent; some studies indicate that genetic diversity is correlated with geographical region, whereas majority does not show such correlations. The divergence might be due to local selection pressure and differences in adaptability of the garlic to different geographical conditions. Recent RAPD and inter-simple sequence repeat findings help to explain the genetic relationships and population structure of garlic accessions of Indian origin.

Keywords: Germplasm, Genetic diversity, RAPD, ISSR, Selection pressures

INTRODUCTION

The rapidly increasing population is placing immense pressure on agriculture for more food. At the same time, agricultural resources are fast being eroded due to traditional farming techniques, urban expansion, pollution and shifting climate patterns. Despite the splendid progress in crop productivity, even greater progress must be made in order to feed billions of people around the world.

Biotechnology, in the simplest and broadest sense, is the utilisation of living organisms or their components to provide useful products or processes and is a dynamic new force for the improvement of crop productivity. Through this, plants can be manipulated to yield more food from the same amount of land, thus preserving the land resources. Production of insect/disease-resistant crops helps in possible reduced use of pesticides, thereby, creating a more pollution-free environment. The ability to grow crops in previously barren pieces of land through the introduction of drought/salinity-tolerant crops would help in increasing the productivity and also feed the millions of hungry mouths, especially in the developing countries of the World. Production of qualitatively rich food like "golden rice" with enhanced nutritional levels, induction of longer shelf life in fruits and vegetables are a few other commendable achievements of agricultural biotechnology.

History of Agriculture in India

Vedic literature provides some of the earliest written records of agriculture in India. Rig-Veda hymns, for example, describe ploughing, fallowing, irrigation, fruit and vegetable cultivation. Other historical evidence suggests rice and cotton were cultivated in the Indus Valley, and ploughing patterns from the Bronze Age have been excavated at Kalibangan in Rajasthan. Some archaeologists believe rice was a domesticated crop along the banks of the Indian River Ganges in the sixth millennium BC. So were species of winter cereals (barley, oats and wheat) and legumes (lentil and chickpea) grown in Northwest India before the sixth millennium BC. Other crops cultivated in India 3000–6000 years ago include sesame, linseed, safflower, mustards, castor, mung bean, black gram, horse gram, pigeonpea, field pea, grass pea

(khesari), fenugreek, cotton, jujube, grapes, dates, jackfruit, mango, mulberry and black plum.

Importance of Vegetables

Vegetables form the most important component of a balanced diet owing to their precious nutritional components: vitamins, minerals, antioxidants and a series of micronutrients indispensable for human health (Bozzini, 2002). Moreover, they can be grown all the year round and provides good and steady income to the growers. In the combat against hunger, vegetables are playing a more important role than before due to the vast diversity present among vegetable species and their ability to survive in almost all terrains. India is the second largest producer of vegetables in the world next only to China, India shares about 14% of the world output of vegetables from about 2.0% of cropped area in the country. Vegetables play a major role in Indian agriculture by providing food, nutritional and economic security and more importantly, producing higher returns per unit area and time. In addition, vegetables have higher productivity, shorter maturity cycle, high value and provide greater income leading to improved livelihoods. With a production of less than 20 million tonnes before independence, vegetable production has increased manifold to 133.7 million tonnes in 2009-10. The area under vegetable cultivation has increased from 5593 thousand ha. in 1991-92 to 7985 thousand ha. in 2009-10.

Among vegetables, Alliums are perhaps the most important species consumed throughout the world. The genus *Allium* comprises approximately 600 known species distributed over Europe, North America, North Africa and Asia. The number of species belonging to the genus has grown steadily over the time as botanist has discovered new species throughout the world. The general characteristics of the *Allium* species are that the plants are almost exclusively herbaceous, perennials and usually form bulbs. Some species, however, often forms thickened rhizomes. In genus *Allium*, Garlic (*Allium sativum*) and Onion (*A. cepa*) are mostly consumed throughout the world. Especially in India, no culinary preparation is complete without onion or garlic. Apart from being used as a prime vegetable, garlic also has numerous medicinal properties. In India, references about cultivation of onion and garlic are found from ancient times onwards as is evidenced by mention in *Charaka-Samhita,* a famous early medical treatise of our country. The varied medicinal uses and pharmacological actions like the anti-inflammatory, antihelmintic and heart friendly nature of garlic were known to ancient Hindus many centuries ago and seem to be supported by modern research (Kirtikar and Basu, 1975).

Garlic is an annual, vegetative grown crop, which can be sown year round in mild climates, in cold climates; cloves can be planted in the ground about 6 weeks before the soil freezes and harvested in late spring. There are different types or subspecies of garlic, and are most notably hard neck garlic and soft neck garlic. Right kind of garlic has to be recommended for a given latitude, since it can be day-length sensitive. In general, hard neck garlic is generally grown in cool climates, while the soft neck is grown close to the equator. Garlic species are classified in to four groups: *A.longicuspis*, *A.ophioscorodon*, *A.sativum*, *A.subtropical* and *A.pekinense* sub-group. The *longicuspis* group is considered the oldest and it is postulated to be the original group. The *ophioscorodon* group is distributed in Central Asia, the *sativum* group in the Mediterranean zone and the *subtropical* in the south and southeast of Asia. Finally, the *pekinense* group comes from the east of Asia. Garlic shows great morphological diversity in bulb size and colour, leaf length, growth habits and agronomic traits such as stress and drought tolerance.

Taxonomy

Bentham and Hooker (1880) had initially classified Alliums under the tribe Alliaceae of the family Liliaceae, on account of its superior ovary. Later, Takhtajan (1967) included this under the order Amaryllidales, family Alliaceae, subfamily Alloideae and tribe Allieae. However, Hutchinson (1934, 1973) and Traub (1968) placed it under the tribe Allieaea of the family Amaryllidaceae, due to the bulbous nature and umbellate inflorescence. It was Dahlgren *et al.* (1985) and Walter *et al.* (1999) who gave Alliums its present status under the family Alliaceae and order Asparagales. Molecular analysis of the genus *Allium* within the Alliaceae was initially done by Klass (1998) through phylogenetic positioning of a plastid DNA sequence coding for the large subunit of ribulose-1-5-bis-phosphate carboxylase. Garlic enjoys a wide

distribution worldwide and has long been known as a cultivated species. Its native home and its existence as a truly wild species are still not clear and have been discussed by Regel (1875, 1887) and Candolle (1885). According to Vavilov (1951) and Vvedensky (1946), *A. longicuspis* Rgl., an endemic species of central Asia, is the wild ancestor of garlic.

The work of Maab and Klaas (1995) too suggests west to middle Asia to be the primary centre of origin and the Mediterranean area as the secondary centre of origin of garlic. However, their work suggests that the garlic of the subtropical regions is different from the other varieties of the crop and probably originated independently a long time ago from *A. longicuspis* group, perhaps in northern India. Jones and Rees (1968) have suggested that the wild ancestor of garlic was flowering and produced seeds or aerial bulbils under different soil and climatic conditions in the ancient civilisation leading to the origin of different varieties gradually. The present day non-flowering varieties are believed to have originated as a result of continued human interference with the natural life cycle caused by storage.

At least two distinct botanical varieties of garlic are recognised, *A. ophioscorodon* and *A. sativum* (Jones and Mann, 1963). Under ideal growing conditions, *A. ophioscorodon* variety is characterised by an initially coiled, tall woody scape and relatively few brownish-purple cloves per bulb. Variety *A. sativum* produces a weak flower stalk, if it bolts at all, and is characterised by a bulb with many pure white or pink-blushed cloves (Engeland, 1991).

Most cultivated alliums have the basic chromosome count of x=8. *A. sativum* is a diploid with 2n=16 (Jones and Mann, 1963; Mc Collum, 1987; Figliuolo *et al.*, 2001). However, some species show a basic chromosome count of 7 and a few species even have 9 as their basic chromosome number (Jones and Mann, 1963).

Morphology and Development

A. sativum L. is a monocot having flat leaves, slender scape, long-beaked spathes and heads bearing bulbils (Kirtikar and Basu, 1975). The small bulbs are always mixed with the flowers of the inflorescence and the flowers usually abort at the bud stage. Though there are reports on the seed production in the literature (Konokov, 1953), confirmation is needed that the garlic ever produces seed (Jones and Mann, 1963). Commonly, garlic is vegetatively propagated through cloves (Jones and Mann, 1963; Figliuolo *et al.*, 2001). Cloves are the sole organs of storage and these are the modified axillary buds of the foliage leaves. At maturity, the main stem of the bulb, roots and leaves attached to it all die. Only the cloves remain to carry the plant on to the next season (Jones and Mann, 1963).

The clove consists of two mature leaves and a vegetative bud. The outer most leaf is the *protective leaf*, which becomes thin, dry and dead at maturity. Within the protective leaf is the single storage organ, the *storage leaf.* Within and at the base of the storage leaf are several very small leaves, which form the bud for next year's growth. The outer most leaf of the bud, the *sprout leaf,* lacks a blade and when the clove sprout, pushes up through the soil, but grows no further. From within the sprout leaf arises the foliage leaves, which constitutes the green top of the plant (Jones and Mann, 1963).

A wide range of adaptability to soil types, temperatures and day length makes garlic farming possible from tropics to temperate latitude (Figliuolo *et al.*, 2001). It is grown worldwide in nearly all cool regions (Novak *et al.*, 1986). Garlic is usually grown as a winter crop in India, i.e., during *rabi* season. It grows vigorously and matures in the spring or midsummer, depending upon the locality and cultivar. Temperature and day length play a very critical role in bulb induction in garlic. Exposure of dormant cloves or young plants to temperatures of 0–10 °C, for one or two months and long days are a pre-requirement for bulb induction. Plants that are never exposed to temperatures below 20 °C fail to produce any bulbs, upon maturity, even under long days (Jones and Mann, 1963).

Chemistry of Garlic

Garlic bulbs are rich sources of carbohydrates and proteins. Analysis of garlic indicates that it contains 61–64% moisture, 31% carbohydrate, 5–6% protein and only 0.2% fat. Significant levels of phosphorous (3.9–4.6 mg/g), potassium (1.0–1.2 mg/g) and calcium (0.5–0.9 mg/g) are present (Kaufmann *et al.*, 1999).

All *Allium* species produce volatile chemicals, which act as repellants to many insects. Garlic has some other chemicals as additional protection for those insects and animals not deterred by its volatile metabolites. Sequestered in vacuoles within the plant's cells is an odourless, sulphur-based compound, (+) *S*-allyl-1 cysteine sulphoxide or alliin (Figure 1).

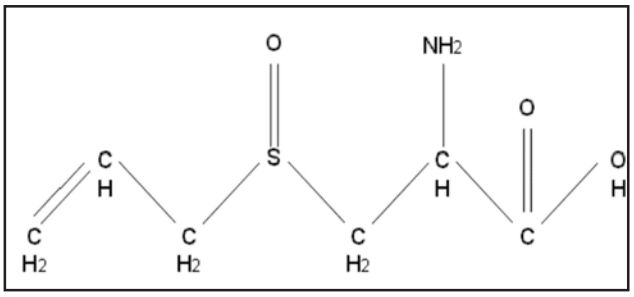

Figure 1: Structure of Alliin

Alliin comprises about 0.24% of the fresh weight of the bulb. In the cytosol, normally separate from alliin, is an enzyme, allinase. When the cell is disrupted, the cell's vacuoles are broken and alliin mixes with allinase resulting finally in the formation of allicin a strong smelling fiery tasting chemical that repels almost every animal. Allicin soon breaks down into diallyl disulphide, which is another strong smelling compound and has been shown to be a powerful insecticide (Kaufmann *et al.*, 1999).

Garlic (*A. sativum*) is one of the important bulb crops grown and used as a spice or condiment throughout India. It is also important foreign exchange earner for India. It is consumed by almost all people who take onion. Garlic has higher nutritive value than other bulb crops. It is rich in proteins, especially in green garlic. Nutritive composition of fresh peeled garlic cloves and dehydrated garlic powder is as follows phosphorous, potassium, calcium, magnesium and carbohydrates. Ascorbic acid content is very high.

Nutritive Value of Garlic

Particular	Fresh peeled garlic cloves	Dehydrated garlic powder
Moisture (%)	62.80	5.20
Protein (%)	6.30	17.50
Fat (%)	0.10	0.60
Mineral matter (%)	1.00	3.20
Fibre (%)	0.80	1.90
Carbohydrates (%)	29.00	71.40
Energy K. Cal	145.00	—
Calcium (%)	0.03	0.10
Phosphorus (%)	0.30	0.42
Potassium (%)	—	0.70
Magnesium (mg/100 g)	71.00	—
Iron (%)	0.001	0.004
Niacin (%)	—	0.70
Sodium (%)	—	0.01
Copper (mg/100 g)	0.63	—
Manganese (mg/100 g)	0.86	—
Zinc (mg/100 g)	1.93	—
Chromium (mg/100 g)	0.02	—
Vitamin A (IU)	0.40	175.00
Nicotinic acid (mg/100 g)	0.40	—
Vitamin C (mg/100 g)	13.00	12.00
Vitamin B (mg/100 g)	16.00	0.68
Riboflavin B2 (mg/100 g)	0.23	0.08
Thiamin (mg/100 g)	0.06	

Source: Vegetable crops in India T.K. Bose and M.G. Som (Ed.) & NIN, Hyderabad

Cultivation of Garlic

The time of planting differs from region to region. It is planted from August to October in Madhya Pradesh, Maharashtra, Karnataka and Andhra Pradesh, and from September to November in Northern plains of India. In Gujarat, planting of garlic is done during October–November. In higher hills, planting of garlic is done during March–April. Cloves of 8–10 mm diameter since give increased yield of better quality, care should be taken to select bigger cloves from outer side of bulbs. About 500 kg cloves of 8–10 mm diameter are required to plant one hectare. Cultivation of garlic can be done either on an annual or biennial basis, depending on whether cloves (annual) or brood bulbils (biennial) are harvested. Depending on weather and climate, rows of bulbils are set from March to April with 4 dt/ha (deciton/hectare) or 355 lbs/acre in rows 20 cm wide with a planting depth of 5 cm. Garlic responds very well to organic manures. For a normal soil, 50 tonnes of farmyard manure, 100 kg N, 50 kg P and 50 kg K/ha through chemical fertilizer has been recommended. Micronutrients also increase its yield potential.

In general, garlic needs irrigation at 8 days intervals during vegetative growth and 10–15 days during maturation.

As the crop matures (when the tops first begin to break over or become dry), it is advisable to stop irrigation to allow field to dry out first. Continued irrigation as the crop matures causes the roots and bulb scales to rot. This discolours the bulbs and exposes outer cloves and decreases the market value of bulbs. Irrigation after long spell of drought results in splitting of bulbs. Excessive irrigation results in sprouting.

Many operations are performed for getting mature and quality bulbs from the field to the consumer. About 15–50% losses occur if proper post harvest management practices are not followed. These practices differ from place to place. Proper curing, sorting and grading, transportation and storage are essential to minimise these losses.

Post Harvest Processing

Drying and curing

Drying and curing are very essential. Drying is done to remove excess moisture from outer skin and neck to reduce storage rot, while curing is an additional process of drying to remove the excess moisture and to allow the colour development and help the bulbs to become compact and go into dormant stage. It is done for about a week in the field for drying. The method and period of curing vary depending on the weather at the time of harvesting. Bulbs are covered along with their tops to avoid damage to bulbs from sun. These are also cured for 7–10 days in shade either with tops or after curing the tops by leaving 2.5 cm above the bulbs and removing the roots. Harvesting at 100% neck fall and curing by windrow method have been recommended. The curing in field till foliage turns yellow should be done. Artificial curing can be done by passing hot air at 27.35 °C through the curing room. It takes about 48 h for complete curing process if humidity is between 60 and 75%.

Sorting and grading

Garlic bulbs after curing are run over a grader or graded manually before their storage or marketing. The thick-necked, splitted, injured and diseased or bulbs with hollow cloves are sorted out. Size grading is done after sorting. It is very much necessary for getting better price and to minimise losses on account of driage and decay. Government of India has prescribed certain grade designations for different qualities of garlic for export. The grade designations and definition of different qualities of garlic have been prescribed.

Packaging

In India, garlic bulbs are packed in open mesh jute bags for domestic use. It is packed in bags of 90 and 40 kg capacity each in Andhra Pradesh, Karnataka and in other garlic growing states, respectively. As per the garlic grading and packing rules, 18 and 25 kg packing are done in perforated 10 ply corrugated cardboard boxes for export. Nylon-netted bags used for packing and further storage cause minimum losses in storage. In foreign countries, plastic-wooven bags are very commonly used. These have good strength and are also attractive. Since garlic needs less ventilation compared to onion, there is a need to develop suitable packaging to reduce driage loss.

Storage

Thoroughly cured garlic bulbs are stored well in ordinary well-ventilated rooms. Garlic with dried leaves can be stored by hanging in well-ventilated rooms. This is, however, not possible on commercial scale because space requirement is more. Storage without tops in nylon-netted bags give better performance at Nasik and Karnal as such the same has been recommended for storage to minimise loss. In Jamangar area (Gujarat), some pockets of Indore and Mandsaur, Madhya Pradesh, and Mainpuri and Etah district of Uttar Pradesh, bulbs are stored for 6–8 months. Since garlic stores well for market under a wide range of temperature, controlled conditioned (low temperature) storage are not necessary. Cloves sprout quickly at 4.4 °C and prolonged storage in this temperature range should be avoided. Storage at 0.5 °C is satisfactory, but high humidity's often accompany low-temperature storage. Garlic stored at humidity higher than 70% at any temperature develop mould and start rotting. Cold storage of garlic is possible at 32–36 F and 60–70% relative humidity. The storage loss of 12.5% is recorded in garlic stored at 1–5 °C and 75% relative humidity compared to 42.4% losses in ambient temperature. Ultraviolet light treatment for 30 min further reduces loss to 8% in cold stores for 150 days storage.

Irradiation with 2–6 Kr of cobalt 60 gamma rays have also been recommended for controlling sprouting in storage. The irradiation given to bulbs within 8 weeks of harvesting (before sprout initiation) can inhibit sprouting effectively, reduce weight loss and can prolong storage life for about 1 year. Doses higher than 10 Kr reduce diallyl disulphide content, which gives typical garlic flavour. Pre-harvest spraying of 0.1% carbendazim and disinfection of premises for handling and storage of garlic also reduce post harvest losses, particularly decay loss. Phosphorus and potassium application reduce weight loss in garlic storage, while nitrogen application increases it.

Garlic is grown in the Northern parts of the country especially in Gujarat, Haryana, Punjab, Maharashtra, etc. and in a few southern localities, especially the cold hilly tracts of Ooty and Kodaikanal, Tamil Nadu. The major garlic growing state is Madhya Pradesh followed by Gujarat.

State-wise Area and Production Data for Garlic

State	Area ('000 ha)	Production (in '000 MT)	Yield (Ton/ha)
Andhra Pradesh	0.40	4.00	10.00
Assam	9.00	39.00	4.33
Bihar	4.30	4.00	0.93
Chhattisgarh	1.00	2.50	2.50
Gujarat	35.90	250.10	6.97
Haryana	3.50	26.10	7.46
Himachal Pradesh	3.60	1.80	0.50
Jammu & Kashmir	0.30	0.30	1.00
Karnataka	5.70	5.80	1.02
Kerala	0.20	0.80	4.00
Madhya Pradesh	54.00	241.50	4.47
Maharastra	3.50	34.10	9.74
Manipur	0.20	0.05	0.25
Meghalaya	0.30	1.10	3.67
Mizoram	1.30	5.60	4.31
Nagaland	0.10	0.20	2.00
Orissa	11.00	35.80	3.25
Punjab	3.70	40.50	10.95
Rajasthan	25.00	150.00	6.00
Tamil Nadu	0.20	1.50	7.50
Uttar Pradesh	32.80	175.90	5.36
Uttaranchal	1.20	7.30	6.08
West Bengal	3.50	33.90	9.69
Total	**200.70**	**1,061.85**	

Data Source: Directorate of Economics and Statistics, Krishi Bhawan, New Delhi, Horticulture Division, Ministry of Agriculture, Govt. of India.

Medicinal Properties of Garlic

Garlic is endowed with many medicinal properties. The bulb is pungent; heating, oleaginous; tonic, aphrodisiac, fattening, digestive, antihelmintic; improves appetite, voice complexion; useful in the diseases of the heart, low fevers, bronchitis, inflammation, piles, leucoderma, asthma, "vata", lumbago, tumours, epileptic fits, thirst and earache, according to Ayurveda. It is a diuretic, carminative, alexipharmac, aphrodisiac; useful in the inflammation, paralysis, pain in the body and the joints, troubles of the spleen, liver and lungs; clears the voice; good for lumbago, chronic fevers, caries of the teeth, leucoderma and thins the blood, according to Yunani (Kirtikar and Basu, 1975).

Louis Pasteur (1858) provided the first modern scientific report of garlic's action as an antibacterial agent (Kaufmann *et al.*, 1999). Garlic is a stimulant, diaphoretic, expectorant, diuretic and tonic. It is rubefacient when applied externally. It has a foul characteristic smell due to the presence of unsaturated sulphides. The juice of garlic is traditionally used for various ailments of the stomach including amoebic dysentery (Shasany *et al.*, 2000). Its use as an anti-tubercular, anti-epileptic, anti-fertility drug and in reducing the blood sugar levels has been reported. The usefulness of garlic in lowering tissue lipids, its antibiotic/antibacterial and an insulin-like effect on the human body has also been reported by Augusti (1990). Its biological activities include antibiotic, anticancerous, antithrombotic and lipid-lowering cardiovascular effects (Fujiwara and Natata, 1967).

Allicin present in aqueous extract of garlic reduces cholesterol concentration in the human blood. The inhalation of garlic oil or garlic juice has generally been recommended by doctors against the cases of impotency, cough and red eyes diseases. In many countries, garlic has been used as traditional medicine, especially to cure several diseases such as Tuberculosis (TBC), influenza, diabetes and high blood pressure. Evidence continues to point to anticancer properties of fresh garlic extracts, aged garlic, garlic oil and a number of organosulphur compounds generated by processing of garlic. These anticarcinogenic and antitumorigenic characteristics appear to arise through both dose- and temporal-related changes in number of cellular events involved with the

cancer process, including those involving drug metabolism, immunocompetence, cell cycle regulation, apoptosis and angiogenesis.

The ability of garlic and related allyl sulphur compounds to block tumours in colon, lung, breast and liver suggested general mechanisms that are not tissue specific. Whereas relatively few studies have compared the relative efficiency of water soluble and lipid soluble allyl sulphur compounds those that have when using chemically induced carcinogen models suggest little difference in response, whereas tumour proliferation/ apoptosis is highly dependent on the species provided. A shift in sulphydryl groups, alterations in glutathione: oxidised glutathione ratios and resultant changes in cellular redox status may be involved in some of the phenotypic changes caused by allyl sulphur compounds. Such changes in thiols by allyl sulphurs may also account for the observed hyperphosphorylation of specific cell cycle proteins and the histone hyperacetylation that has been correlated with suppressed tumour cell proliferation. In agriculture, garlic is used as bactericide and fungicide.

Biotechnological Approaches in Improvement of Garlic

Common garlic cultivars have a somatic chromosome number of 2n=16 (with a karyotypic formula of six metacentric chromosomes, four submetacentric chromosomes and six acrocentric chromosomes), although some garlic plants found in the Campania region of Italy were shown to be tetraploid (4n=32), some cultivars might be triploid. Chromosomal aberrations are common in garlic, due to multiple translocations involving 8 or even 10 chromosomes. Some sterile cultivars have a normal karyotype (see in Jo *et al.,* 2012) Therefore, in garlic breeding programmes, genetic variation can be increased only by somaclonal variation, mutagenesis or genetic transformation. Characterisation of the garlic germplasm has largely been based on phenotypic characteristics; however, morphological characteristics can vary under different agroclimatic conditions.

Because of its asexual nature, new forms by conventional methods of breeding are difficult to achieve. This has become an impediment for developing new varieties in garlic. Biotechnological approaches such as tissue culture is important in development of new verities in garlic mostly through somatic embryogenesis and meristem culture. Research in and application of tissue culture technology can be divided conveniently into five broad areas, namely: (a) Cell behaviour, (b) Plant modification and improvement, (c) Pathogen-free plants and germplasm storage, (d) Clonal propagation and (e) Product formation. In some areas, the advances have been made or the potential remains unrealised.

Plant tissue culture is a technique in which isolated plant cells, tissues, organs or even whole plants are grown in nutrient medium under aseptic conditions. The differentiation of garlic callus into whole plant using three clones has been described (Kehr and Shaeffer, 1976), but these authors did not report the frequency of regeneration from any of the clones. The regeneration in garlic involving both organogenesis and embryogenesis was initially reported by Abo El-Nil (1977). Regeneration of protoplast into whole plant from one clone through a callus stage was demonstrated by Ayabe *et al.* (1995). The best callus induction media for the leaf explants (with epidermis) was MS (Murashige and Skoog) medium supplemented with 3 mg of 2,4-D+2.2 mg of IBA/l (Li *et al*., 1999). Optimum callus induction from leaf explants was achieved on MS medium supplemented with 2 mg of IAA, 0.5 mg of 2,4-D and 0.1 mg/l of kinetin (Maggioni *et al*., 1989). Yun *et al*. (1998) reported MS medium to be an effective medium for shoot multiplication and bulb let production from shoot tip explants. Barandiaran *et al.* (1999) evaluated regeneration in 20 garlic clones after 3 months of culture. A simple protocol for garlic *in vitro* management is described. It comprised a single medium for all developmental stages and used immature bulbs as source of axillary buds.

A simple and efficient garlic *in vitro* shoot regeneration protocol has been developed by Barandiaran *et al.* (2004). This system uses axenic root tips cultivated from the beginning in the presence of light and does not require any change or refreshing of the original medium during the entire process. The application of light from the beginning of the culture process did not affect the callus formation rate, but did significantly improve the explant regeneration ability. In a 2-month period it was possible to obtain up to 250 shoots per gram of callus. Reports of somaclonal variation among garlic regenerates are limited. Novak (1980) reported variation in range of phenotypic characters including plant height, umbels,

bulb weight, shape and number of cloves within a bulb and evaluated regeneration of long-term callus cultures using one clone and found that organogenetic potential decreased and genetic instability increased.

Tapia (1987) explored somaclonal variation as a means of obtaining improved garlic forms, whereas Vidal *et al.* (1993) found a somaclone possessing consistently higher bulb yield than the parental clone. Some workers tried to detect somaclonal variations through RAPD and cytological analysis and concluded that no association existed between the rate of variation for molecular and cytological characters either by comparing cultivars or examining individual regenerate and also suggested that the frequency of variation is cultivar dependent. *In vitro* garlic flowering was achieved in a liquid medium, which included 100 p.p.m. GA_3 and 100 p.p.m. tetracycline. Bhojwani (1980) was the first to test different hormonal regimes for multiple shoot regeneration through direct organogenesis in *A. sativum* using 0.1 mg/l of NAA and 0.5 mg/l of 2-iP. In this method of micropropagation, adventitious shoots arise directly from the tissues of the explants and do not develop within previously formed callus (George and Sherrington, 1984). Single cytokinenin source without any supplementation of auxin successfully promoted multiple shoot culture in *Allium*. Initially, Kahane (1992) introduced 5 µM BA as the only plant growth regulator (PGR) for initiation of multiple shoots, the sufficiency of 0.5 mg/l of BA for the same task in *A. tuberosum*. Haque *et al*. (1997) reported direct organogenesis for the first time from root tip. Haque *et al*. (1998) in *A. sativum* recognised the competency of BA alone in direct organogenesis.

Nagakubo *et al.* (1993) was the first to report the *in vitro* bulblet formation directly from excised shoot tips of *A. sativum* inoculated in LS basal medium. Successful bulblet production in B5 medium supplemented with 8% sucrose in garlic. A higher sucrose supplementation (12%) in MS medium regenerated bulblets directly from root tip explants was done. Roksana *et al*. (2002) inoculated surface-sterilised individual clove of *A. sativum* in MS medium plus 0.5 mg/l of 2-iP along with 0.25mg/l of NAA to regenerate multiple bulblets. Multiple bulblet formation was induced by repeated (fourth) subculture of regenerated plantlets on both liquid and semi-solid MS medium. The regenerated bulblets were successfully established in soil .Supplementation of 10µM JA in liquid MS medium with 0.1 mg/l of NAA and 11% sucrose helped in direct bulblet regeneration from root tip of *A. sativum* in a better frequency was reported by Kim *et al.* (2003).

Regeneration of shoots from an unorganised mass of tissue produced from any explants is termed as indirect organogenesis. These later gave distinct bulbils from the base of the differentiated plantlets. Nagasawa and Finer (1988) for the first time successfully demonstrated the callus initiation and proliferation using MS media plus 0.3 mg/l of 2,4-D in *A. satvium*. This report of efficacy of 2,4-D as the only PGR for callus culture was further favoured by several successors in *A. ampeloprasum* (Buiteveld *et al.*, 1993) and in *A. sativum* (Al Zahim *et al*., 1999; Robledo *et al.,* 2000). Robledo *et al.* (2000) reported efficient plant regeneration of garlic by root tip culture. Root apices from *in vitro* cultured garlic cloves were used as axenic explants for organogenic callus production and plant regeneration experiments. 2,4-D (2.2–4.5 µM) alone or combined with kinetin (2.3–4.6µM) were used to induce organogenic callus. Addition of a lower level of Cytokinin with a comparatively higher level of 2,4-D resulted in enhancement of callus culture than 2,4-D alone. Introduction of BA or BAP as the alternative cytokinin source with 2,4-D proved best for *A. ampeloprasum* and *A. cepa* was reported. Due to its sexually sterile nature garlic breeding has been limited to clonal selection of landraces or spontaneous mutants. However, development of new garlic cultivars has become increasingly feasible now by sexual hybridisation. Genetic transformation or development of somaclonal variants depends upon the availability of highly efficient callus production and regeneration system. Regeneration via somatic embryogenesis from calluses of bulb, leaf disc, stem tip and leaf can be done. Regeneration could be either through organogenesis or embryogenesis.

In garlic, it was observed that regeneration occurred via organogenesis and embryogenesis in most cases and Abo E1-Nil (1977) was the first to report this kind of regeneration. Later, this mode of regeneration was reported by many others (Xue *et al.,* 1991; Masuda *et al*.,1994, Bockish *et al.,* 1997), Haque (1998) established a competent practice of plant regeneration from root tip of *A. sativum* via somatic embryogenesis in MS medium containing 0.5 mM kinetin. Al Zahim *et al.* (1999) studied

the regeneration through somatic embryogenesis from long-term calluses of five garlic cultivars. Fereol *et al*. (2002) established a unique scheme for somatic embryogenesis and plant regeneration in *A. sativum* using young leaf or root explants from *in vitro* plants as the sources of explants. They reported that the embryogenic potential was higher in callus proliferated from young leaves in B5 medium supplemented with 0.1 mg/l of 2,4-D and 0.5 mg/l of Kn. The regeneration of somatic embryos to plants with shoots and roots was observed on BDS medium with 0.3mg/l of BAP.

Zheng *et al*. (2003) worked on the development of an efficient cultivar-independent plant regeneration system from callus derived from both apical and non-apical root segments of garlic. Callus induction and later plant regeneration were studied in four widely grown garlic (*A. sativum* L.) cultivars from Europe. Root segments from *in vitro* plantlets were used as starting material. Callus induction on apical root segments was significantly higher compared to callus induction on non-apical root segments in the second series of experiments. Two months after callus induction, callus lines were transferred to a regeneration medium consisting of MS basal medium supplemented with 30 g/l of sucrose and 1 mg/l of (4.6 mM) kinetin. Regeneration via somatic embryogenesis from calluses of bulb, leaf disc, stem tip, leaf, receptacle and flower bud and direct organogenesis or embryogenesis from shoot tip or stem disc explants in garlic has been reported by various authors and summarised by Khar *et al.* (2003).

A study concerning the effect of 2,4-D on indirect somatic embryogenesis and surface structural changes in garlic (*A. sativum* L.) was conducted to evaluate its effect on the induction of somatic embryogenesis and to observe the developmental stage of somatic embryo as well as the surface structural changes of somatic embryo in garlic. Root tip explants were cultured on embryogenic callus induction medium (ECIM). Somatic embryos were then transferred to the embryo maturation medium (EMM), desiccated and subsequently transferred to the repetitive somatic embryogenesis medium (RSEM). Observation by dissecting microscope showed that embryogenic callus and somatic embryo was formed at ECIM containing 0.1 µM 2,4-D. At EMM containing 0.01 µM 2,4-D, somatic embryo developed into mature somatic embryo. Somatic embryo underwent repetitive somatic embryogenesis that consisted of globular and mature somatic embryo at RSEM, without PGR.

A lower level of 2,4-D (0.25 mg/l) with comparatively higher level of BAP (1 mg/l) supplementation in MS medium significantly affected or even they boost up the rate of regeneration from somatic embryo in *A. sativum.*

Diversity Studies in Garlic

Genetic diversity is required for populations to adapt to environmental changes. Large populations of naturally out breeding species usually have extensive genetic diversity, but it is usually reduced in populations and species of conservation concern. Assessment of genetic diversity at the molecular level is more meaningful than at the phenotypic level as the later involves data on morphological traits, which are environmental dependent. Though they significantly contribute towards phenotypic variation but cannot be accurately phenotyped. So the study of polymorphism is best done at the level of arrangement of nucleotide bases in DNA, the primary source of all biological information. At this level, even seemingly identical accessions could display enormous differences, if only we could employ appropriate DNA profiling techniques. Molecular tools provide valuable data on diversity through their ability to detect variation at the DNA level. Identification is of fundamental importance in diversity studies in a variety of different ways. For evaluation of species diversity, it is essential that individuals can be classified accurately. The identification of taxonomic units and endangered species, whose genetic constitution is distinct from their more abundant relatives, is important in the development of appropriate conservation strategies. In the population studies, molecular tools like molecular markers or genetic markers are being used to identify whether the two individuals are from the same species or are from the specific parents and estimating the degree of relatedness among individuals.

A genetic marker is a trait used as a marker of genetic variation within and among individuals and taxa. Traits used include phenotypic traits, protein products and segment of the DNA. DNA-based molecular markers have acted as a versatile tool and have found their own position in various fields like taxonomy, physiology, embryology, genetic engineering, etc. A large number of

DNA-based molecular markers have been developed, of which hybridisation-based restriction fragment length polymorphism marker technology was first and widely used in research. But being costly, labour intensive and time consuming, a more simple technique amenable to automation was looked for.

Pooler and Simon (1993) worked on morphological diversity of garlic and observed the differences in bulb and leaf size, scape presence, height, colour of flowers, fertility and bulbil (top set). Simon and Jenderek (2003) have reported wide range of morphological diversity in garlic, including flowering ability, leaf traits, bulb traits, plant maturity, bulbing response to temperature and photoperiod, cold hardiness, bulbil traits and flower traits. Kamenetsky *et al.* (2004) revealed that cultivar characteristics differ considerably with the location of cultivation. Climate can have a significant impact on garlic flower stalk formation and also on the taste and variety.

Genetic and molecular studies in garlic are challenging due to its large genome size and, until recently, a strictly asexual life cycle for the cultivated crop. Kirk *et al.* (1970) have reported that garlic genome has a low GC base composition of 36.9% and a high amount of repetitive DNA. Lack of flowering in most cultivated clones and seed sterility in those that do flower have also restricted the sexual breeding and genetic studies in garlic. DNA content of garlic is 32.5 pg 2C nucleus, which is one of the largest genome among the cultivated crops and only slightly smaller than that of the onion (33.5pg/2C; Ranjekar *et al.*, 1978). Although no authentic evidence of polyploidy in garlic has been presented (2n=2x=16), extensive intra-chromosomal duplication has been suggested in Alliums by Jones and Rees (1968) and King *et al.* (1998).

Various types of molecular markers are utilised to evaluate DNA polymorphism and are generally classified as hybridisation-based markers and polymerase chain reaction (PCR)-based markers. In the former, DNA profiles are visualised by hybridising the restriction enzyme-digested DNA, to a labelled probe, which is a DNA fragment of known origin or sequence. PCR-based markers involve in *in-vitro* amplification of particular DNA sequences or loci, with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermos table DNA polymerase enzyme. The amplified fragments are separated electrophoretically and

Table 1: Selected list of work done by the authors on Garlic

S.No.	Author	Title
1	Etoh *et al.* (2001)	RAPD variation of garlic clones in the centre of origin and the westernmost area of distribution.
2	Volk *et al.* (2004)	Genetic diversity among US garlic clones detected by using AFLP (amplified fragment length polymorphism) methods.
3	Panthee *et al.* (2006)	Diversity analysis of garlic (*A. sativum* L.) germplasms available in Nepal based on morphological characters.
4	Kucera *et al.* (2007)	Genetic diversity among garlic (*A. sativum* L.) clones as revealed by AFLP, phenotypic descriptors and S-Amino acid level.
5	Zahedi *et al.* (2007)	Evolution of Iranian garlic genotypes using multivariate analysis method.
6	Buso *et al.* (2008)	Genetic diversity studies of Brazilian garlic (*A. sativum* L.) cultivars and quality control of clover production.
7	Ipek *et al.* (2008)	Rapid characterisation of garlic (*A. sativum* L.) clones with locus-specific DNA markers.
8	Khar *et al.* (2008)	Analysis of genetic diversity among Indian garlic (*A. sativum* L.) cultivars and breeding lines using RAPD markers.
9	Nair *et al* (2011)	Inter-simple sequence repeat (ISSR) marker-based analysis for genetic diversity in Indian garlic
10	Nair *et al* (2011)	Assessment of genetic diversity in Indian garlic using RAPD markers
11	Jabbes *et al* (2011)	ISSR fingerprints for assess genetic diversity of Tunisian garlic populations
12	Singh *et al* (2011)	RAPD profile-based grouping of garlic germplasm with respect to photoperiodism

banding patterns are detected by different methods such as staining and autoradiography.

Lallemand *et al.* (1997) carried out study in a collection of clones coming from 25 countries by using biochemical markers determined low isozyme variability for 65 evaluated clones. Apparently, the low variability between garlic groups was only due to the presence of one or a few mutations, which were not accompanied by important changes in the rest of the genome. AFLP technique has also been used to characterise garlic by Hong *et al.* (2000), Lampasona *et al.* (2003) and Volk *et al.* (2004). Choi *et al.* (2003) analysed 75 clones of garlic and classified them in two large groups. The first group was formed by the Asian clones and the second one by the European, American and Russian clones.

RAPD technique is a well-accepted tool, which is commonly utilised in genetic studies to detect total genetic variation within and among populations. The technique has been successfully used in several taxonomic and genetic diversity studies. The ease of employing this technique has facilitated its use in the analysis of genetic relationship in several instances. There are some points of apprehension regarding RAPD-generated phylogeny and these include: bands homology showing the matching rate of migration, causes of variation in fragment mobility and origin of sequence in the genome. Despite these precincts, RAPD marker has some advantages: able to scan across all regions of the genome, and therefore, highly appropriate for phylogenetic studies at species level.

Volk *et al.* (2004) reported that 64% of the US National Plant Germplasm System's garlic collection held at the Western Regional Plant Introduction Station in Pullman, Washington, USA, and 41% of commercial garlic collections were duplicates. Detection and elimination of those duplicated accessions in germplasm collection can reduce maintenance costs significantly. Germplasm from a country of origin may help to aid in the selection of appropriate growing climates. The premise behind this is that a selection will have developed in adaptation to a particular climate, making it better suited to similar climates (Fernandez *et al.,* 2003). Prior studies have used total genomic DNA to screen for molecular markers by employing such method as RAPD's (Nabulsi *et al.,* 2001; Ipek *et al.,* 2003). RAPD analysis has been successfully applied to interspecific classification of number of crop species, in the genus *Allium*. Close relations of *A. cepa* have already been analysed with RAPD markers by Wilkie *et al.* (1993).

MaaB and Klaas (1995) used isozymes and RAPD markers to clarify older classification scheme, to see the extent of genetic diversity in different groups and to gauge the effect of domestication, adaptation and the spread to different climatic condition on the loss in diversity. Based on the result they proposed an interspecific classification of garlic into four major groups and *A. longicuspis* to be the progenitor of *A. sativum*. RAPD technique is employed to assess genetic variation in garlic and examine relationship between cultivated garlic and the wild progenitor *A. longicuspis*.

Molecular marker studies in garlic have not been very exhaustive because of its asexual nature. RAPD technique has been widely used for characterisation of Australian garlic by Bradley *et al.* (1996). Shasany *et al.* (2000) classified 21 Indian garlic genotypes along with Argentinean clones and reported a diversity of 60% by RAPD analysis, whereas a diversity of 20% only was assessed by morphological features. Xu *et al.* (2001) confirmed that the RAPD technique is a useful and efficient tool in classification and identification of garlic genetic resources.

Kamenetsky *et al.* (2005) worked on the analysis of diversity in fertility potential and organo-sulphur compounds among garlics from central Asia. RAPD analysis was used to evaluate genetic diversity among eight garlic mutants resistant to white rot disease by Nabulsi *et al.* (2001). Mutants characterised with moderate resistance to white rot were closely related to the control using cluster and correlation analyses. On the other hand, highly resistant mutants were quite distant from the control with low correlation coefficients.

Panes *et al.* (2004) described genetic diversity of Philippine *A. sativum* L. using RAPD primers. Results of the morphological data analysis based on analysis of variance showed that the six morphological features are significantly different at 0.5 level of significance. Genetic diversity studies of Brazilian garlic cultivars and quality control of garlic-clover production was carried out by Buso *et al.* (2008). Results revealed that the number of

markers were efficient and sufficient to obtain a coefficient of variation of 10%. Similarity varied between 16 and 98% and cluster analysis showed that, in general, genetic similarities correlate with morphological characters of the cultivars and production cycle variation.

Genetic diversity of eight selected Argentinean garlic clones were investigated at DNA level using AFLP procedure by S. Garcia, Lampasona and J.L. Burba (2003). Variation in productive characteristics and diversity assessment of garlic cultivars and lines using DNA markers was studied. RAPD and ISSR markers were assayed to determine the genetic diversity of sex garlic lines and three garlic cultivars.

Maintenance of redundant garlic (*A. sativum* L.) accession is expensive due to the necessity of yearly regenerating garlic accessions in germplasm centres. Therefore, rapid characterisation of garlic accession is important for avoiding duplicate genotypes.

Hernandez *et al.* (2008) compared the yield and genetic relationships between two Perla garlic selections, obtained by individual selection of cloves, and commercial varieties and cultivars adapted to diverse regions of Mexico. Varieties with fewer cloves showed higher yields. The selection method used to obtain plants with better yield characteristics can be applied to the genetic improvement of garlic. Abdoli *et al.* (2009) carried out classification of Iranian garlic ecotypes using 10 RAPD primers. No significant relationship between genetic diversity was detected by RAPD technique and geographical origins. Evaluation of some agronomic traits and genetic relationships among developed garlic clones by RAPD markers and protein analysis was carried out by Abdhikader-Helmy *et al*. (2011). The results of agronomic traits for the clones showed relatively wide range of genetic variability among garlic genotypes and showed great potential for improving agronomic traits in garlic.

In Indian context, except for one or two reports on molecular characterisation of Indian garlic germplasm by RAPD technique, not much work has been done. A lot of efforts towards diversity assessment of Indian garlic are important. Beside this with the reports of flowering garlic, it has become imperative on our part to import those lines and start breeding work on introduction, adaptation and development of segregating families for further breeding work towards yield improvement and resistance breeding.

ISSRs has recently been developed as an anonymous, RAPD-like approach that accesses variation in the numerous microsatellite regions dispersed throughout the various genomes and circumvents the challenge of characterising individual loci that other molecular approaches require. They are characterised by mono, di- or multi-nucleotide repeats that have 4–10 repeat units side-by-side. Extremely high variability combined with greater robustness in repeatability experiments and less prone to changing band patterns with changes in constituent or DNA template concentrations, make them superior to other readily available marker systems in investigations of genetic variation (Petolescu and Nedelea, 2009).

ISSR primers target simple sequence repeats (microsatellites) that are abundant in eukaryotic genome and evolve rapidly, but they do not require any prior knowledge of DNA sequence for primer design reported by Fang and Roose (1997). Zietkiewicz *et al.* (1994) added that ISSR can rapidly differentiate closely related individuals, Fang and Roose (1997), Dagani *et al.* (2003), Salhi Hannachi *et al.*(2004, 2005) and Okpul *et al.* (2005) have reported that ISSR markers have been successfully used to assess genetic diversity among closely related cultivars, which were difficult to distinguish with other molecular markers. ISSR-PCR has also been used to fingerprint the different plant species and cultivars.

Very less work has been carried out in Alliums genetic diversity using ISSR markers. The genetic diversity of 32 onion (*A. cepa* L.) germplasm resources were analysed by ISSR primers by Xu *et al.* (2007), which indicated a rich genetic diversity of onion (*A. cepa* L.) germplasm resources. According the result of Unweighted Pair Group Method with Arithmetical Averages (UPGMA) cluster analysis indicated that ISSR technique is useful in revealing the genetic diversity and genetic relationship among onion germplasm materials, providing a scientific basis for genetic breeding and patents selection.

Ma *et al.* (2009) characterised eight novel polymorphic

simple sequence repeat (SSR) markers, developed from an enriched genomic library of garlic (*A. sativum* L). Phenogram was constructed to understand the relationships among the 90 accessions. These newly developed SSRs should prove very useful tools for genotype identification, assessment of genetic diversity and population structure in garlic. Gantait *et al.* (2010) worked on the determination of genetic integrity of long-term micropropogated plantlet of *A. ampeloprasum* L. using ISSR markers. Detection of genetic integrity for *in vitro* regenerated clones was carried out using ISSR primers.

Very recently, Cunha *et al.* (2012) have developed new microsatellite markers for garlic. A new set of SSR markers for garlic, an important medicinal spice, was developed to aid studies of genetic diversity and to define efficient strategies for germplasm conservation. The new SSR markers have the potential to be informative tools for genetic diversity, allele mining, mapping and associative studies, and in the management and conservation of garlic collections.

Two most recent studies have appeared, the one deals with Indian garlics (Nair, 2012). This author has used 160 RAPD primers, of which only 40 primers gave amplification and 20 primers gave good amplification, 189 bands were generated and 65 were polymorphic (34.39% polymorphism). OPE 08 showed maximum polymorphism (63.63%) and OPM 09 showed minimum polymorphism (12.5%). Further, of the 100 ISSR primers screened only 27 gave amplification; of which 14 were good. In all, 123 bands were generated and 45 were polymorphic (36.58% polymorphism). Primer 825 (62.5%) and 808 (60%) showed maximum polymorphism, whereas primer 834 showed least polymorphism (20%). The UPGMA dendrogram constructed using Jaccard's similarity matrix of RAPD data and discriminated germplasm in two major groups: Group I had four sub-clusters (Ia, Ib, Ic and Id), Ia and Ib showing 100% similarity, while Ic and Id showed 85% similarity. Group II comprised of one Sub-Cluster and showed 89% similarity. Group 1 and 2 showed 74% similarity when compared. ISSR data discriminated all the germplasm into two major groups. Group 1 comprising of five sub-clusters (Ia, Ib, Ic, Id and Ie). Ia, Ib and Ic showed 100% similarity, while Id and Ie showed 91% similarity. Group 2 consisted of two clusters and showed 90% similarity among them. Groups 1 and 2 showed 79% similarity between them. When RAPD and ISSR similarity matrix compared they showed GS value ranging from 0.57 to 0.33.

In summary, of the 96 morph types, 16 types were identified as genetically divergent from the rest. Total number of bands varied from 186 with RAPD and 112 with ISSR .There was 100% similarity with both the primers. Banding pattern was clear, consistent and excellent. The studied morphotypes displayed enormous morphological diversity for several characters, but the molecular markers exhibited low genetic diversity in garlic germplasm. Total lack of sexual reproduction could be the reason for low genetic diversity. Garlic crop was domesticated nearly 5000–6000 years ago, the extent of genetic or chromosomal mutations did not appear to have contributed significantly for creating genetic variability. From among 96 types, 16 types are genetically distinct for various agronomic traits: purple colour: 8; single clone and red colour: 4; red bulbs: 4. It is inferred that most morphs are genetically related/there are duplications in the germplasm bank at Pune.

High numbers of bands were detected indicating polymorphism but had low genetic diversity. Most of the polymorphic bands showed low frequency. It is proposed that two selection pressures – one for morphology and second for genetic traits worked independently in evolution of garlic's morphotypes.

Jo *et al*. (2012) have classified the genetic variations of garlic. Seven selected SSRs revealed a total of 37 alleles across 120 garlic accessions, with an average of 7 alleles per locus. The values for observed heterozygosity ranged from 0 to 0.99 (mean=0.71). The average genetic diversity and polymorphic information content values were 0.586 and 0.518, respectively. Based on the phylogram constructed, the garlic accessions were clustered into four main groups (G1–G4) in the phylogram. Group 1 consisted of accessions of 'Aomori', Group 2 consisted of 64 accessions, Group 3 consisted of 25 accessions and Group 4 consisted of 20 accessions. These workers correlated genetic diversity with geographical region. There may have been local selection pressure and differences in adaptability of the garlic to different geographical conditions. All of

the tested loci deviated significantly ($P<0.01$) from Hardy–Weinberg equilibrium. Thus, a number of disturbances occurred in the garlic population tested, including natural selection. These investigations help in explaining the genetic relationships and population structure of garlic accessions.

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