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Phenotypic and Genetic Diversity of South Indian Allium Sp. (*A.cepa* And *A.sativum*) by Molecular Fingerprinting To Select The Superior Germplasm

KEYWORDS: Allium sp, DNA fingerprinting, Genetic diversity, Molecular markers, RAPD, UPGMA

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ABSTRACT *Allium is a genus of vegetative propogation, showing high morphological diversity and genetic diversity and its analysis plays an important role in breeding programs. The objective of this study is to determine the phenotypic and molecular polymorphism of onion and garlic collected from different cultivation sites. Among 20 random primers used, seven primers which produced the highest number of bands were used for DNA amplification. The proportion of polymorphic bands per total number of bands detected was higher for O1 (13.3%) compared to that of O2 (12.9 %) and O3 (5.79%). The proportion of polymorphic bands per total number of bands detected was higher for G1 (46.37%) species compared to that of G3 (38.7 %) and G2 (22.4%). From this study, we conclude that O2 and G3 are the superior germplasm in onion and garlic cultivars respectively.*

Introduction

Plant diversity is very important for the stability of excellence atmosphere, flimsy ecosystems, current/future universal well-being and superior safekeeping. They provide valuable traits necessary for adapting plants to varying climatic conditions, biotic and abiotic stresses and outbreak of pandemic diseases. Plants cater many human needs such as food, fuel, fibre, oil, herbs, spices, medicine and wood for furniture, paper pulp, industrial crops and as forage and fodder for domesticated animals. Despite ~ 27000 plant species around the world, studies have estimated that only 30 crops provide 95% of human food energy needs with just four of them, viz., rice, wheat, maize and potatoes, provide more than 60%. With even tremendous medical advances, only 20% of the world's plant species have ever been tested for their medical potential.

Given the significance of a relatively small number of crops for global food-health security, it is critically important to conserve the diversity within these plants. The confront of feeding a world population which is growing steadily lies on a handful of domesticated crop plants and will necessitate an astonishing increase in food production. Studies project that, by 2050, world population will increase from the existing level of about 6 billion to more than 8 billion people (Hoisington *et al.*, 1999). It is sketchy that humankind will require to produce as much food for the duration of the next 50 years as was produced since the commencement of agriculture 10,000 years ago (Hoisington *et al.*, 1999). Thus, there is vital need to recognize superior populations, quickly differentiate and select elite contender(s) and breed new varieties for achieving current as well as future food and global fitness security needs (Singh *et al.*, 2008).

Allium is one of about 570 genera of flowering plants with more than 500 species (Frodin, 2004). It is by distant the largest genus in the Amaryllidaceae, and also in the Alliaceae in classification system in which that family is documented as separate (Rahn, 1998). Studies of genetic diversity in onion have been hampered by a lack of portable codominant molecular markers (McCallum, 2007). The phenotyphic and genotyphic variability of onion and garlic was studied by Singh *et al.* (2010) and Dubey *et al.* (2010) respectively. Although a variety of molecular marker methods have been successfully used to resolve questions of genetic diversity and relatedness to species level in *Allium* (Klaas & Friesen, 2002), identifying robust and informative markers within *A.cepa* has proved much more challenging. Dominant randomly applied polymorphic DNA markers have been used in limited studies of *A.cepa* diversity (D'Ennequin *et al.*, 1997; Tanikawa *et al.*, 2002), but a more detailed evaluation by Bradeen & Havey (1995) showed that identification of reliable, heritable polymorphism is very challenging in onion.

The categorization of garlic germplasm has been based mainly on phenotypic characteristics. However, morphological characteristics can vary under diverse agroclimatic circumstances. This situation adds convolution to the characterization of garlic clones (Bradley *et al.*, 1996; Al-zahim *et al.*, 1997). To eliminate some of these limitations, biochemical (Pooler & Simon, 1993; Lallemand *et al.*, 1997) and molecular markers (Wilkie *et al.*, 1993; Bark & Havey, 1995; Maab & Klaas, 1995; Dubouzet & Shinoda 1999; Garcia Lampasona *et al.*, 2003; Volk *et al.*, 2004; Rosales-Longo *et al.*, 2007; Ovesna *et al.*, 2007; Ovesna *et al.*, 2011; Mukherjee *et al.*, 2013) have been used in genetic diversity studies of this species. The objective of this study was to determine the genetic diversity of *Allium* genus (*Allium cepa* and *Allium sativum*) collected from various agroecological environments by means of RAPD (Random Amplified Polymorphic DNA).

Materials and Methods

Collection of sample

The onion cultivars used in this study was collected from different cultivation sites; Surandai (O1) and Alankulam (O2) in Tirunelveli district and Vilathikulam (O3) in Thoothukudi district. Garlic clones were collected from Poomparai (G1), Mannamannar (G2) from Kodaikanal and Vadugapatti (G3) from Theni District. The clones were subjected to the assessment of various phenotyphic and genetic diversity studies.

Assessment of phenotypic characters

The quantitative phenotypic characters such as height and width of the leaf, bulb and flower stalk, dry and wet weight of the bulb were determined. Every character was repeated five times.

DNA Isolation

The genomic DNA isolation was done as per the protocol 200mg of the root sample was taken and it was grind to a fine powder with mortar and pestle using liquid nitrogen. 1ml of ice cold nuclei extraction buffer in the w/v ratio of 1:5 in 1.5ml vial was added and kept the resuspended ground powder on ice for 20 mins, repeated above steps for the next samples Spin 20 mins at 2700g at 4^0C. The supernatant was poured off and the pellet (contains nuclei) was stored. 0.8ml

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of nuclei lysis buffer was added and gently resuspended the pellet by inverting the tube several times gently and incubated at 65^{0}C for 20-30 mins in water bath. Equal volume of chloroform:isoamyl alcohol was added and inverted several times gently to remove proteins. The tubes were centrifuged for 5 mins at 2700g at RT to separate the aqueous and organic phase. The above step chloroform:isoamyl alcohol step was repeated to the collected supernatant. The aqueous layer was taken and the DNA was precipitated by adding 0.6 Vol of Ice cold Isopropanol. Gently mixed to aggregate DNA, spooled out DNA and washed it twice in 70% Ethanol. The pellet was air dried and the DNA was resuspended in 100-200µl of sterile distilled water in an 1.5ml vial .The solution may be warmed briefly several times to 65^{0}C to resuspend the DNA (Doyle & Doyle, 1987). The DNA extracted was analyzed on 1.0% agarose gel and DNA quantification was done using Nanodrop.

RAPD Fingerprinting

The genomic DNA was diluted accordingly, reaction mixture contains Template-4ul(15ng) 10x Taq assay buffer 1.5µl, MgCl2:0.3µl, Primer: 5µl (1pmoles/µl) and Taq polymerase: 0.3µl the reaction volume was made up to 15µl with water and amplification was performed in a programmable Thermal Controller (*Eppendorf personal master cycler)* for RAPD fingerprinting, an initial denaturation of 4 min. at 94°C , followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 38°C for 1 minute, and extension for 2 minutes at 72°C. A final extension for 7 minutes at 72°C was included after the last cycle. Amplification was done using random primers. The high range DNA marker contains 12 DNA fragments ranging from 0.5kbp to 10 kbp and the fragments are as 0.5,0.6,0.8,1.0.1.5,2.0,3.0,4.0,5.0,6.0,8.0,10kb p. The number of monomorphic bands, number of polymorphic bands, and intensity of bands were recorded. Primers, which on amplification produced the maximum number of bands, were used to amplify the DNA and the amplification profile using seven primers were taken with the help of gel documentation system. The polymorphic bands were represented as '+' (present) and '–' (absent). The seven primers that produced polymorphism in onion clones were OPA01, OPN05, OPF07, OPG12, OPC15 OPE09 and OPY18. The seven primers that produced polymorphism in garlic clones were OPA01, OPN05, OPF07, OPG12, OPC04 OPE09 and OPY18. The amplification profiles of those primers, which produced amplification for most of the varieties, were used in the final analysis. Based on their scoring matrix the data was subjected to analysis using *MVSP-32* version RAPD analysis software. The genetic similarity matrix was calculated using Jaccards co efficient, distance matrix and the dendrogram was based on Eucledian distances are obtained using UPGMA (Unweighted Pair Group Method with Arithmetic mean).

Primers used for RAPD fingerprinting:

OPG12 – CAGCTCACGA OPY18 – GTGGAGTCAG

OPA01 – CAGGCCCTTC OPF07 – CCGATATCCC

OPE09 – CTTCACCCGA OPC15 – GACGGATCAG

OPN05 – ACTGAACGCC OPC04 - CCGCATCTAC

Phylogenetic Analysis

The distance matrix and the dendrogram using UPGMA was done using MVSP 32 RAPD software program.

Results

The analysis of the phenotypic characters such as height and breadth of the leaves, bulbs, wet and dry weight of the bulbs and number of bulbs, size of the bulbs does not showed any markable difference between the different onion and garlic clones. The qualitative characters of *Allium* clones revealed no major differences (Table 1). The height and diameter of the bulb of *A.cepa* ranges from 3.02 to 3.68 and 1.68 to 5.34 respectively for O1 and it was found to be larger when compared to other two clones. The wet and dry weight of the bulb also found high in O1 (Wet weight – 2.74 to 28.89, dry weight – 0.56 to 4.71). The length and breadth of the leaf was found high in O3 (23.5 to 45.8 and 0.44 to 1.02). The height and diameter of the single bulb of *A.sativum* ranges from 3.06 to 4.48 and 0.9 to 1.92 respectively for G2 and this was found to be larger than other two garlic clones. The wet and dry weight of the bulb and the size of the leaf were varied with the garlic clones (Table 1).

Table1. Phenotypic characters of *Allium cepa* and *Allium sativum* after harvesting

Cultivar	Length of the bulb (cm)		Diameter of the bulb (cm)		Wet weight of bulb (g)		Dry Weight of bulb (g)		Height of the leaf (cm)		Width of the leaf (cm)	
	Small	Big	Small	Big	Small	Big	Small	Big	Small	Big	Small	Big
O1	3.02±0.25	3.68±0.42	1.68±0.13	5.34±0.50	2.74±0.49	28.89±7.05	0.56±0.09	4.71±1.08	18.6±2.04	43.6±5.86	0.5±0.07	0.74±0.09
O2	2.9±0.29	4.04±0.35	1.76±0.29	4.84±0.49	3.01±0.86	24.16±3.60	0.554±0.15	3.76±0.64	15.1±3.21	45.2±1.89	0.56±0.11	0.76±0.17
O3	2.54±0.39	3.68±0.30	1.86±0.23	4.6±0.43	3.07±0.37	19.43±3.41	0.64±0.11	3.73±0.63	23.5±3.14	45.8±4.27	0.44±0.11	1.02±0.11
G1	2.14±0.21	3.12±0.26	1±0.1	1.72±0.30	0.94±0.18	2.73±0.74	0.38±0.03	1.04±0.23	51.4±2.30	66.8±4.66	1.14±0.17	1.84±0.21
G2	3.06±0.59	4.48±0.31	0.9±0.187	1.92±0.41	0.71±0.29	5.14±0.84	0.29±0.09	1.83±0.26	49±5	68.2±2.95	1.1±0.16	1.46±0.29
G3	2.3±0.25	2.68±0.69	1.08±0.08	1.6±0.1	0.98±0.30	2.62±0.16	0.39±0.08	0.93±0.04	51.8±2.95	63.6±5.41	1±0.16	1.2±0.19

The amount of DNA in onion samples O1, O2 and O3 were 167.89 ng / µg, 222.97 ng / µg and 203.81 ng / µg respectively. The DNA content in garlic clones were 178.67 ng/µg, 157.41 ng/µg and 349.87 ng/µg respectively for G1, G2 and G3 (Table 2).

Table 2. Quantification of DNA from *Allium* clones.

Sample	A.cepa clone			A.sativum clone		
	O1	O2	O3	G1	G2	G3
DNA ng/µl	167.89	222.97	203.81	178.67	157.41	349.87
Absorbance 260/280	1.85	1.89	1.85	1.91	1.87	1.99

In this study, RAPD profile of *Allium cepa* produced 205 total scorable bands out of 7primers examined for *Allium cepa,* of these 25 polymorphic bands were examined. Primer OPG12, OPA01 produced highest number of fragments among the primers used with an average of 44 fragments ranging from 200 bp to 5000 bp while OPF07 produced lowest number of fragments(21) ranging from 500bp to 3000 bp (Fig. 1). Among the primers that generated polymorphic amplification products primers OPA01 produced highest percentage of polymorphism (18.1%) followed by OPE09 produced lowest polymorphism (4.5%). The proportion of polymorphic bands per total number of bands detected was lower for O3 species compared to that of O1 (13.3 %). The percentage of polymorphic bands in O3 was the lowest followed by O1, O2 (Table 3).

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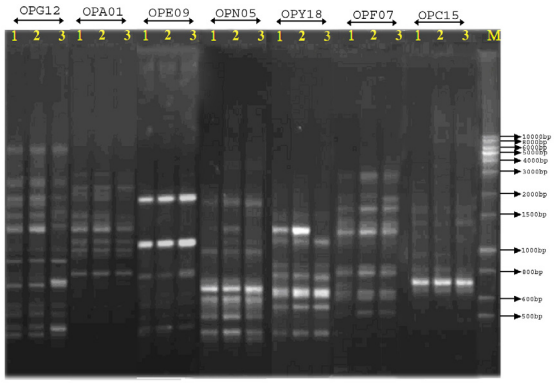

Figure 1. Showing the polymorphic band patterns of *Allium cepa*

Table 3. Showing % of polymorphism in *Allium* **sps.**

Primers	A.cepa clone			A.sativum clone		
	O1	O2	O3	G1	G2	G3
OPG12	3/15	4/16	1/13	4/10	4/10	4/10
OPA01	4/16	4/16	0/12	4/15	1/12	2/13
OPE09	1/8	0/7	0/7	12/13	1/2	13/14
OPN05	0/8	0/10	2/10	4/9	2/7	2/7
OPY18	1/9	1/9	0/8	2/6	2/6	2/6
OPF07	0/7	0/7	0/7	4/8	1/5	1/5
OPC15	1/7	1/7	0/6	-	-	-
OPC04	-	-	-	2/8	0/7	0/7

Among the 3 different *Allium cepa* species O1 and O3 form a cluster. The Euclidean distances are higher between O2 and O3. The similarity coefficient was highest O1 and O3. The dendrogram linked O1 and O3 at a distance while the dendrogram, out grouped O2 from rest of the species (Fig.2). From the dendrogram, we can determine that the O1 and O3 form a clusters and has a linkage distance (1.386) for UPGMA Eucledian distances , while O3 and O2 showed an highest distance (2.594) (Table 4). From this dendrogram we can conclude tentatively that the O1 and O3 are linked in a cluster and are similar varities while O2 form an out group.

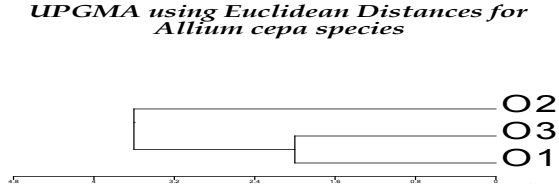

Figure 2. Dendrogram of all the variables of *Allium cepa* using UPGMA

Table 4. Showing the Euclidean distances between *Allium cepa* **clones.**

Cultivar	O1	O2	O3
O1	0	-	-
O2	2.401	0	-
O3	1.386	2.594	0

RAPD profile of *Allium sativum* produced 189 total scorable bands out of 7 primers examined, of these 65 polymorphic bands were examined. Primer OPG12, OPA01 produced highest number of fragments among the primers used with an average of 35 fragments ranging from 600 bp to 3000 bp, while OPY18 produced lowest number of fragments (17) ranging from 200 bp to 2000 bp (Fig.3). Among the primers that generated polymorphic amplification products primers OPE09 produced highest percentage of polymorphism (82.7%) followed by OPC04 produced lowest polymorphism (9.0%).

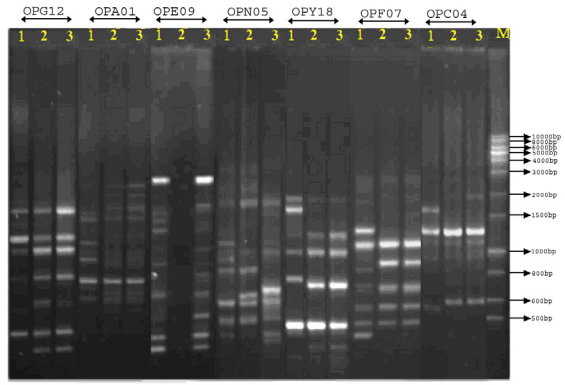

Figure 3. Showing the polymorphic band pattern of *Allium sativum*

The proportion of polymorphic bands per total number of bands detected was lower for G2 species compared to that of G1 (46.37%). The percentage of polymorphic bands in G2 was the lowest followed by G1, G3 (Table 3). Among the 3 different *Allium sativum* species G1 and G2 form a cluster. The Euclidean distances are higher between G1 and G3 (Table 5). The similarity coefficient was highest G1 and G2. The dendrogram linked G1 and G2 at a distance while the dendrogram, out grouped G3 from rest of the species (Fig. 4). In both *Allium* genus, the primer OPG12 was reported to amplify more DNA fragments, ie., between 200 bp and 5000 bp.

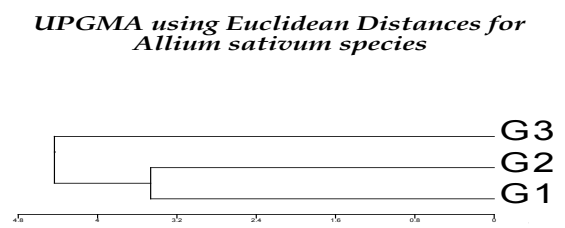

Figure 4. Dendrogram of all variables of *Allium sativum* **using UPGMA**

Table 5. Showing the Euclidean distance between *Allium sativum* **clones.**

Cultivar	G1	G2	G3
G1	0	-	-
G2	3.606	0	-
G3	5.099	3.873	0

4. Discussion

Given the phenotypic plasticity of *Allium* germplasm, it is very difficult to perform a precise classification of plants and bulbs without having an environment carefully controlled by the experimental design. In contrast, genotypes and individual taxa within and between gene-pools could be directly distinguished by analyzing the naturally occurring variation of DNA. In this study, we determined that the cultivars showed no major differences regarding the phenotypic characters. Molecular markers are able to describe the evolutionary

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pathways within and between species. Closely related taxa, including siblings species, that may have gone unrecognized by morphological traits can been distinguished by molecular markers (Avise , 1994). This can be demonstrated by looking at the genetic distances among groups which are linearly related with time since divergence, and can be used to assess the effective genetic separation of a priori defined genepools.

Due to the large number of bands which detected polymorphism (346) in the analysis of Paredes *et al*. (2008), it can be said that the RAPDs gave a good approximation of the genetic relationship existing among the evaluated clones. It is assumed that the amplified DNA fragments (amplicons) that co-migrate in the different accessions are similar in their sequences. Wilkie *et al*. (1993) isolated and analyzed several common RAPDs garlic bands and analyzed their homology by means of a DNA hybridization process under two types of astringency, low and medium-high. Like their study, the result obtained in this study also indicated that all the common RAPDs bands isolated of similar intensity showed high homology.

Mukherjee *et al*. (2013) reported in their study that RAPD produced a total of 408 bands (34 bands per primer) ranging from 200 bp (primer OPAA17) to 6800 bp (primer OPH 1). In this, we reported the size of the amplified bands was ranging between 200 bp and 5000 bp. The primer OPG12 gave the maximum band patterns.

Figliuolo & Di Stefano (2007) reported the RAPD marker UBPZ24 was polymorphic within and between gene-pools with eight distinct fragments ranging from 0.43 to 3 kb. Nonetheless, based on marker UBPZ24, *A.sativum* softneck Italian landrace and Great headed garlic confirmed to be two different clones. Polymorphism for UBPZ24 was observed within the Sardinian wild *A.ampeloprasum* and between two genotypes of single bulb garlic.

Paredes *et al*. (2008) used forty random primers of 10 mers generated a total of 398 bands with an 87% of polymorphism. Each primer amplified between two and 20 bands. The size of the fragments obtained fluctuated between 3200 and 369 bp. Their results showed that the clones analyzed had a genetic similarity rate of 94%. In addition, 70% of them were clustered in one more group. However, in spite of that situation several clones have different agronomic characteristics.

Although there is low genetic diversity as detected by the RAPD, this molecular marker could be used to identify some of these clones. This information could be quite useful to complement the morphologic and agronomic information necessary for the registration of some of these clones, maintain the purity of the variety and protect the breeder's rights of some of the selections. However, to improve the identification process of these clones it is necessary to incorporate another type of molecular markers which would allow the detection of greater genetic differences among clones (Paredes *et al*. (2008).

5. Conclusion

The present study verified that rapid and commercial markers like RAPD could be successfully applied to studying taxonomic relationships at the interspecific and intraspecific level in *Allium* genus. The large number of distinctive bands obtained in the present study signifies the supremacy of RAPD fingerprinting and diversity analysis. From this we can conclude that the varieties that have highest similarity may be phenotypically similar and may share most of the genotypic characters. While the varieties that exhibit lowest similarity may be phenotypically different as well as genotypically they may exhibit some differences. The differences in the phenotypic characters may also be influenced by the environmental and geographical characters. Based on the molecular fingerprinting the onion cultivar O2 and the garlic cultivar G3 were concluded as the superior germplasm among the examined cultivars.

Conflict of interest

None

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