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Onion diversity analysis using morphological and molecular approaches

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ABSTRACT

In this mini review, significance of onion as an important vegetable for world-wide cultures has inadvertently set the tone to genetic diversity among onions and its significance. This diversity study has used five data sets, only to prove their partial utility to properly classify this vast genus *Allium*. These studies have brought out limitations of biochemical (isozyme) data as well as molecular data. These limitations have led to development of techniques/concepts of RAPD, GISH and RFLP, along with refinement in DNA isolation procedures from onion and electrophoretic separation procedures to minimize ambiguities in interpretation of data, be it on an evaluation of cytoplasm N, S and T or evaluation of onion genome from major onion producing countries. This work has thus laid a firm foundation for onion improvement program for inducing traits desired by consumers and for industrial applications.

KEYWORDS

Genetic diversity;
Onion improvement;
Isozyme;
RAPD;
RFLP.

INTRODUCTION

Onions are one of the oldest vegetables known to mankind, used for their flavor, aroma and taste. They are available in fresh, frozen, canned, pickled and dehydrated forms. Depending on the variety, an onion can be spicy and pungent or mild and sweet[1]. They are used in vast number of recipes and preparations, spanning world's almost all cultures. Onions are used, usually chopped or sliced, in almost every type of food, including fresh salads, cooked foods, as a spicy decoration and an accompaniment to the main course. They are used domestically or industrially as a raw material for a variety of food manufacturing processes such as dehydration, freezing, canning and pickling[2].

Onions are cultivated species, though their wild related species are still found in the areas, which are regarded as botanical centers of origin for the crop: the south western part of Central Asia, largely covered by Iran, Afghanistan, Pakistan and Southern Republics of the Soviet Union[3].

GENETIC DIVERSITY

Genetic diversity refers to any variation in nucleotides, genes, chromosomes or whole genome of organisms. This is the fundamental basis and currency of diversity as reflected from approximately 1 billion different genes, recognized from all the known species on earth[4].

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Significance of genetic diversity

During the process of evolution, small advantageous characteristic changes occurring randomly have been passed over from one generation to the next. The genetic characters are observable structures or molecular entities from different species, such as gene products or metabolic pathways. Accurate assessment of genetic characters and their relationship(s) in crop species is an important component of crop improvement programs, as it serves to provide information about genetic diversity. This includes (i) analysis of genetic variability in cultivars, (ii) identification of diverse parental combinations to create segregating progenies with maximum genetic variability for further selection and (iii) introgressing desirable genes from diverse germplasms into the available genetic base.

Analysis of genetic diversity in germplasm collections facilitates reliable classification of accessions with possible utility for specific breeding purposes. Significant emphasis is being paid to comprehensive analysis of genetic diversity in numerous crops, including major field crops such as wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), maize (*Zea mays* L.), barley (*Hordeum vulgare* L.), and soybean [*Glycine max* (L.) Merr.][5].

GENETIC DATA SETS

Genetic diversity could be studied by analyzing the numerical observations on variables of populations and individuals. For this purpose, different data sets have been used by scientists, most important among such data sets are (i) pedigree data[6], (ii) passport data/ morphological data[7], (iii) biochemical data obtained by analysis of isozymes[8], (iv) storage proteins[9] and (v) DNA-based marker data, which allow more reliable and rapid differentiation of cultivars. Since each of these data sets provide different types of information, the choice of analytical method(s) depends on the (a) objective(s) of the experiment, (b) level of resolution required, (c) resources and technological infrastructure available and (d) operational and time constraints, if any[10].

Genetic diversity plays an important role in plant breeding, because hybrids between lines of diverse origin generally display greater heterosis than those between closely related strains. However, the maximum heterosis generally occurs at an optimal or intermediate level of diversity. One of the potent techniques of measuring genetic divergence is the D^2 statistics proposed by Mahalanobis[11]. Subsequently, Rao[12] suggested the application of D^2 statistics for the assessment of genetic diversity in plant breeding. This technique measures the forces of differentiation at two levels namely, intra-cluster and inter-cluster levels and thus helps in the selection of genetically divergent parents for exploitation in hybridization programs.

Genus *Allium*

It is a large genus and contains several major agricultural crops including bulb onion *(Allium cepa),* shallot *(A. cepa* syn. *A. ascalonicum),* Japanese bunching or Welsh onion *(A. fistulosum),* chive *(A. schoenoprasum),* garlic *(A. sativum)* and leek *(A. ampeloprasum* syn. *A. porrum).* All these common/ domesticated *Alliums* have a basic chromosome number of 8 and most are diploids *(A. cepa*: *2n=2x=16)*[13]. Classification of such a large genus has proved difficult and many ambiguities still exist[14].

Initial efforts for classification of Genus *Allium*

Vvedensky[15] classified the cultivated *Allium* species into four sections: Cepa (bulb onion), Phyllodolon (Japanese bunching onion), Porrum (garlic and leek) and Rhizirideum (chive). A later classification based on morphological criteria, crossability and karyotype[16], also divided them among four sections (Allium, Cepa, Fistulosa and Rhizirideum) with further divisions into sub-sections. However, difficulties arose, because of (i) few morphological characters upon which the classification was based and (ii) strong barriers to crossing separate, but morphologically similar species. Therefore, it has been suggested that there could be a role for genetic markers in the systematic study of the *Allium* species[17].

Association of morphological marker(s) with a gene of interest serves as a useful tool for selection. However, morphological markers are affected by (a) dominance and late expression, (b) deleterious effects, (c) pleiotrophy, (d) epistasis, (e) rare polymorphisms, (f) paucity of numbers and (g) non-tight linkages[18].

BIOCHEMICAL DATA USING ISOZYME MARKERS

The work on isozyme markers, started since 40 years, is regarded extremely useful in view of presence of multiple forms of enzymes in plants. The isozymes are separated by starch gel electrophoresis and visualized as bands by substrate-stains on gels. For this purpose, esterase, acid phosphatase, peroxidase, catalase and leucine amino peptidase isozymes in onion seedlings were used[19].

On the basis of allelic forms of isozymes (allozymes), which are co-dominant markers, heterozygous phenotype can be visualized. For example, in rice, strong correlation between isozyme phenotype and characters like photosynthetic ability[20] and seed protein content[21] has been found. However, isozyme markers being limited in number, growth stage and tissue-specific, has limited their use. Isozyme analysis using large *Allium* collections was the first application of molecular markers in onion. It was applied to (i) 29 populations, each with 30-60 individuals of *Allium douglasii*, 188 accessions of *Allium cepa* and 29 accessions of *Allium fistulosum*[22], (ii) 110 accessions of *Allium sativum*[23], (iii) 300 accessions of *Allium sativum*[24] and (iv) 189 accessions of *Allium cepa* var. *ascalonicum* and *Allium wakegi*[25].

MOLECULAR DATA

Molecular markers as a function of natural variation

DNA markers, which occur as a natural variation and are present in large number, provide several advantages over isozyme markers by virtue of genetic information being stored[26]. Although plants carry out replication of this DNA with high accuracy and rapidity, changes in DNA do occur due to various mechanisms operating. These changes may be (i) simple base pair changes or (ii) large scale changes, resulting due to (a) insertion, (b) deletion, (c) duplication, (d) substitution, (e) translocation and (f) transposition[27]. Therefore, various molecular marker methodologies have been utilized to visualize DNA polymorphism[28]. The most common molecular markers currently in use are (i) restriction fragment length polymorphism (RFLP), (ii) random amplified polymorphic DNA (RAPD), (iii) amplified fragment length polymorphism (AFLP), (iv) simple sequence repeat (SSR) and (v) micro-satellite[29].

RAPD (Radom Amplified Polymorphic DNA) markers

Use of RAPD markers to assess in-bred integrity

Commercial onion bulb growers had complained that hybrids grown successfully by them for few years failed to give expected yields. Therefore, RAPD was used for assessing in-bred integrity. The assessment showed that in-breds used to produce hybrid-onion seeds, (i) rarely self-pollinated for more than two generations, (ii) retained high level of heterozygosity and (iii) selection, drift or contamination over the period affected their performance. RAPD markers identified between two in-bred onion lines, were used to examine changes in independently maintained and publically released in-bred onion lines and their Mendelian inheritance demonstrated, which revealed contamination, contributing to lower yields[30].

RAPD in conjunction with morphological markers for assessing genetic relationship

The *Allium cepa* species included two major crops on the basis of their morphological traits and typical reproduction mode: (i) sexually reproduced biennial onions and (ii) vegetatively propagated perennial shallots, which rarely flower. In addition, the seed-propagated shallot, a recently released variety, with an intermediate phenotype for life history, has been used by breeders. These species were analyzed using molecular markers (RAPD) and morphological characters of growth and development. Morphological data recorded from onions and vegetatively propagated shallots was submitted to multivariate statistical analysis. For molecular marker study, European and tropical onion accessions were chosen. Results indicated that seed-propagated shallot was more closely related to onions than to vegetatively propagated shallots, besides a geographical genetic diversity[31].

Use of GISH and RAPD to prove hybrid status

Three vegetative crops of *Cepa* in genus *Allium* (top onion, French grey shallot and viviparous triploid

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onion) of suspected hybridogenic origin were studied, using genomic *in situ* hybridization (GISH) and RAPD markers. The results showed that (i) in *A. x proliferum*, parental chromosomes were derived from *A. fistulosum* and *A. cepa* as unequivocally identified by GISH to prove hybrid status of this crop and (ii) French grey shallot belonged to *A. oschaninii* on the basis of RAPD analysis[32].

Use of RFLP and RAPD to establish origin and relationship between two taxa

The origin of *Allium fistulosum* (bunching onion) and its relation to *Allium altaicum* was examined by (a) RFLP analysis of five non-coding cpDNA regions and (b) RAPD analysis of nuclear DNA. While RFLP analysis could distinguish the two species, only RAPD analysis clarified the inter-relationship between the two taxa[33]. RAPD and PCR-RFLP analysis was also used to establish phylogenetic relationship among collected accessions of shallot and *Allium x wakegi*, as also to assess its origin. The results indicated that (i) out of 100 primers, 20 amplified with 112 scorable bands for cluster analysis, (ii) out of 2 main cluster groups, one group belonged to shallots and another to *A. x wakegi* and (iii) sub-groups of clusters reflected phenotypic differentiation in shallots and regional specificity in some *A. x wakegi* accessions[34].

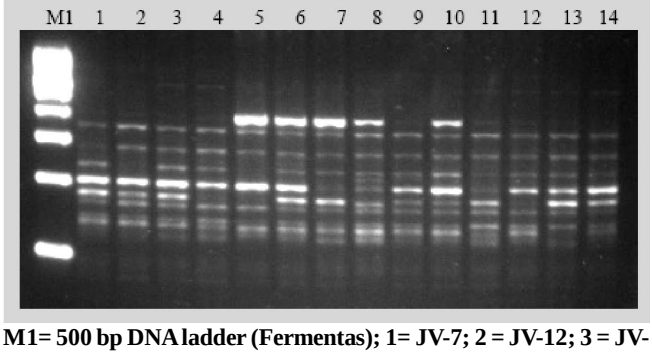
Inter-specific hybridization, performed between wild and cultivated species of genus *Allium* generated hybrids, possessing characteristics of both parental plants, as judged by RAPD analysis[35]. Similarly, inter-specific hybrids ($2n = 16$) between *Allium fistulosum* L. ($2n = 16$) and *Allium schoenoprasum* L. ($2n = 16$) were studied using RAPD. The results confirmed the hybridity[36].

Use of RAPD for assessing genetic diversity among cultivars

RAPD markers were also used to estimate genetic diversity among 24 cultivars of short-day onions. For this purpose, (i) total genomic DNA was extracted and subjected to RAPD analysis using 90 arbitrary decamer primers. Of these primers, (i) 15 selected primers yielded 137 bands, 91.2% of which were polymorphic and (ii) none produced unique banding pattern for each cultivar. RAPD analysis (a) grouped 24 onion cultivars into two major clusters, from the northern region and southern region of India and (b) showed high diversity among the selected onion cultivars[37].

Use of RAPD and field data for assessing genetic diversity among cultivars

Field data involved evaluation of 14 cultivars for morphological characters such as (i) plant height (cm), (ii) number of leaves, (iii) bulb weight (g), (iv) bulb diameter (cm), (v) Total Soluble Solids (%), (vi) bolting (%), (vii) doubles (%), (viii) pungency (ppm) and (ix) yield (tons/acre), using standard statistical procedure. On the basis of mean performance of these cultivars, their clustering was undertaken, which had grouped them into five clusters. RAPD markers were also used to estimate genetic diversity among these 14 cultivars of short-day onions. For this purpose, total genomic DNA was extracted using modified mini-prep protocol of Vorh *et al.*[38], with ease and cost effectiveness. RAPD analysis was performed using decamer primers (Figure 1) to note that (i) these 31 primers yielded 77.2% polymorphic bands and (ii) none produced unique banding pattern for each cultivar. RAPD analysis grouped 14 onion cultivars into four clusters, one cluster with cultivars of exotic origin and other three included cultivars of Indian origin. Comparison of clustering based on RAPD as well as field performance indicated (a) high diversity among the onion cultivars selected, (b) maximum divergence among the exotic and Indian cultivars and (c) potential of RAPD markers for identification / maintenance of onion germplasm for onion improvement[39,40].



M1= 500 bp DNA ladder (Fermentas); 1= JV-7; 2 = JV-12; 3 = JV-16; 4 = Phule Safed; 5 = Arka Kirtiman; 6 = Punjab White; 7 = Agrifound White; 8 = Udaipur-102; 9 =Arka Pitambar; 10 = Pusa White Flat; 11 = Pusa White Round; 12 = Gujarat Local; 13 = ARS-1; 14 = ARL-2

Figure 1 : Amplification RAPD profiles of 14 onion ultivars with primer OPD-05

RFLP (Restriction Fragment Length Polymorphism) markers

This technique, picked from the tool box of molecular geneticists, has been available since about 15 years.

Use of RFLP to distinguish N, S and T cytoplasms

In onion too, RFLP was used to detect polymorphism between sterile and fertile cytoplasms. *Bam*HI and *Hind*III digestion of mtDNA distinguished among N-, S- and T- cytoplasms. Undigested mitochondrial DNA did not produce plasmid-like molecules on gels, indicating that S-cytoplasm in onion does not possess small circular DNA molecules, similar to S-cytoplasm in maize (*Zea mays*)[41].

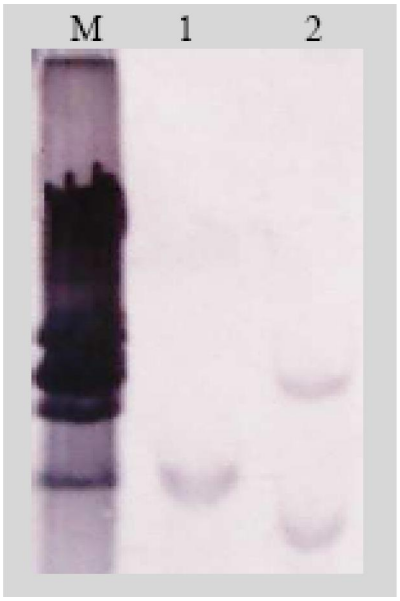
Evaluation of onion genomes from major producing countries

Populations of bulb onion (*Allium cepa*), collected from Middle East, Central Asia and Indian sub-continent, were evaluated for the presence of normal (N) male-fertile and male-sterile (S) cytoplasm, using three polymorphisms in the chloroplast genome. It revealed prevalence of N cytoplasm among populations originating from Central Asia and confirmed it to be wild type[42].

As some confusion had surfaced from the early studies on source(s) of CMS extracted from the indigenously open-pollinated onion populations, male sterility was evaluated in onion plants from USA[43], Germany[44], Turkey[45], New Zealand[46], Holland[47] and India[48]. Male sterility from Dutch cultivar Rijnsburger was assumed to be S-cytoplasmic[47]. However, since it broke down at high temperature, it was concluded to be T-cytoplasmic[49].

Using RFLP analysis as per Havey[49], Adsul[39] has confirmed N, S and T types of cytoplasm in cultivars of Indian origin and in some exotic cultivars (Figure 2). Further, sterility in two Indian cultivars was confirmed to be different from S type male sterility.

Even though RFLP marker system is quicker to crosses, it is still relatively time-consuming and labor-intensive compared to PCR, which is significantly quicker and cheaper method of evaluating DNA polymorphism[50].



M = λDNA/ *Eco*RI digest (Genei, Bangalore); 1 = Male sterile; 2 = Male fertile

Figure 2 : RFLP analysis of onion mitochondrial DNA for male sterility using *CoxI* as a probe

Use of RFLP and RAPD for development of onion genetic map

A low-density genetic map of morphological markers, RAPD and RFLP was developed as a tool for (i) studying the genome organization of onion and (ii) its improvement. For example, (a) a mapping population of 58 F_3 families was produced from a single F1 plant from the cross of two partially in-bred lines (*Brigham Yellow Globe* 15-23 and *Alisa Craig* 43) and (b) segregations (14 RAPDs, 110 RFLPs) were established for restoring male fertility in sterile cytoplasm and complementary light-red bulb colour[51].

Advantages of RFLP technique are (i) its requirement of small quantity (5-10 μg) of DNA, (ii) membranes can be reprobed, (iii) multiple filters corresponding to individual samples can be simultaneously probed to produce linkage maps, (iv) most of the markers are co-dominant as isozymes, (v) right DNA probe picks up differences (polymorphism) among the plants and (vi) its capability to detect virtually unlimited differences.

CONCLUSION

Onion diversity studies assisted with molecular approaches have helped in clear classification of *Allium* species and thus of great help in onion improvement activities.

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Conventional onion breeding has history of over a century, while molecular approaches have started recently. Though conventional breeding has resulted into some successes, it is time-consuming and labour-intensive. Onion breeding using molecular approaches is still only a distant possibility. In this context, it is highly desirable to use molecular data to assist conventional onion breeding.

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