# RAPD Analysis of Ornamental Alliums for Phylogenetic Relationship

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# RAPD Analysis of Ornamental *Alliums* for Phylogenetic Relationship

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## Introduction

*Allium* is a large genus that has approximately 700 species (Huxley et al.[5]); which are predominantly found in dry, rocky terrain in the northern, Mediterranean and temperate regions with distinct seasonal variations. *Allium* includes several major agricultural crops; common onion (*A. cepa*), chives (*A. schoenoprasum*), garlic (*A. staivum*), Chinese chives (*A. tuberosum* Rottler ex Sprengel syn. *A. odorum*), Japanese bunching onion (*A. fistulosum*), and leek (*A. ampeloprasum*). Besides, it includes many ornamental species; *A. schubertii*, *A. giganteum*, *A. cowanii*, and *A. moly*. All the common cultivated *Alliums* have a basic chromosome number of 8 and most of them are diploid.

The taxonomy of such a large genus as *Allium* is very difficult. Vvedensky[10] classified the cultivated *Alliums* into four sections, *Cepa* (common onion), *Phyllodolon* (Japanese bunching onion), *Porrum* (garlic and leek) and *Rhiziridium*. More recent classification schemes based on morphological criteria, crossability, and karyotype, have also divided them into four sections, *Allium*, *Cepa*, *Fistulosa* and *Rhiziridium*, with further divisions into subsection (Traub[9]). Isozyme polymorphism has also been used to study interspecific relationships in *Allium*. Four sorts of isozyme were tested for the analysis of intraspecific differentiation in *A. wakegi* (Okubo and Fujieda[7]). However, the major limitation of isozyme analysis is insufficient polymorphisms prevalemt among the closely related cultivated plants. Traditional identification based on morphological traits requires extensive observations of mature plants and, frequently, it lacks definition and objectivity for those who are not taxonomists. Furthermore, morphological traits sometimes change serve as unambiguous markers because of environmental influences.

In recent years, a valuable contribution to plant improvement programs was made, particularly by polymorphic DNA markers based on restriction fragment length polymorphisms (RFLPs), which have the potentials to reveal an almost unlimited number of polymorphisms. Havey[3] estimated phylogenetic relationships among the cultivated *Allium* species utilizing RFLPs of the chloroplast DNA. Some disadvantages of RFLPs are the laborious procedure involved, relatively high cost, and use of radioisotopes, thus the RFLP procedure presents certain problems in case of the relatively large and complex genomes such as *Allium*.

As an alternative, the random amplified polymorphic DNA (RAPD) technique developed by Williams et al.[15] and Welsh and McClelland[12] provides a faster and easier approach for exploring genetic polymorphism, requiring only a very small amount of DNA as well. The RAPD technique has been used in a lot of crops including *Allium*. Wilkie et al.[14] estimated the

phylogenetic relationships among *Allium* species by using RAPD and placed *A. roylei* as the closest relative of *A. cepa*.

In this study, the genomic DNA of *Allium* species was extracted, then RAPD markers were amplified, using arbitrary 10-mer primers through the polymerase chain reaction (PCR). RAPD analysis was applied for detecting polymorphisms among the ornamental *Alliums*. The specific object was to determine the potential for amplification of polymorphic markers in ornamental *Allium* speciese for genotype identification, phylogenetic analysis, and the future development of a genetic map of *Allium*.

## Materials and Methods

### 1. Plant materials

Ten species of the genus *Allium* grown in a greenhouse (Tabel 1) were tested to distinguish the species each other by RAPD markers. The bulbs of those materials were planted in November of 1993. The leaves were sampled out of the plants counting from 3 to 5 per species.

Table 1. Species used in this study

| Species | Abbreviation | Flowering Season | Flower Color |
|---|---|---|---|
| *A. tuberosum** | T | Year-round | White |
| *A. cowanii* | C | Spring | White |
| *A. giganteum* | G | Spring | Purple |
| *A. schubertii* | S | Spring | White-Pink |
| *A. unifolium* | U | Spring-Summer | Pink |
| *A. albopilosum* | Al | Summer | Purple |
| *A. ampeloprasum* | Am | Summer | Light Pink |
| *A. caeruleum* | Ca | Summer | Blue |
| *A. moly* | M | Summer | Yellow |
| *A. sphaerocephalum* | Sp | Summer | Purple |

*: cv. Tender Pole

### 2. Isolation of Genomic DNA

The SDS (sodium dodecyl sulfate) extraction method described by Watanabe and Sugiura[11] was modified by the addition of one washing step to remove the phenolic compounds during DNA extraction. The young leaves (0.15g) of these accessions were cut into small slices, which were then placed in a 1.5 ml microcentrifuge tube and ground with a plastic pestle of Kontes until no large pieces could be seen. A 500 $\mu\ell$ extraction solution (15% saccharose, 50 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 5 mM NaCl) was added to it and then were centrifuged for 5 min at 1,000g at 4℃. The precipitate was mixed with washing solution (0.1 M N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 0.1% polyvinylpyrrolidone, 4% 2-mercaptoethanol), and then was centrifuged for 5 min at 15,000g at 4℃ two or three times. The precipitate was suspended in 300 $\mu\ell$ 2T-1E (20 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0), and 40 $\mu\ell$ of SDS was added to each tube and the tubes were incubated for 15 min at 70℃. After cooling to room temperature, ammonium acetate was added to a final concentration of 1.5 M and then the solution was kept for 30 min in ice, followed by a centrifugation of 15,000g

for 15 min at 4℃. The DNA from the supernatant was a sort of sediment with equal volume of isopropyl alcohol and this was kept for 15 min at room temperature, and then it was centrifuged for 15 min at 15,000g at room temperature. Isopropyl alcohol residues were removed by adding the cooled 70% Et-OH two or three times. After evaporating the Et-OH, the DNA precipitate was redissolved in TE pH 8.0 (10 mM Tris-HCl, 10 mM EDTA,). After adding RNase to a final concentration of 1 μg/ml, the resuspended DNA was incubated for 1 hour at 55℃. DNA concentration and purity were estimated by UV spectrophotometer and then adjusted to a final concentration of 10 ng/μℓ by TE.

3. RAPD-PCR conditions

Nine arbitrary 10-mer primers (OPD-01, OPD-03, OPD-05, OPD-07, OPD-08, OPD-12, OPD-15, OPD-18, OPD-20) from Operon Technologies (Alameda, USA) were used to amplify the RAPD fragments. Reactions were carried out in a final of 25 μl containing 50 mM KCl; 10 mM Tris-HCl pH 8.8; 0.1% Triton X-100; 2.0 mM $MgCl_2$; 25 ng template DNA; 0.2 mM each of dATP, dCTP, dGTP and dTTP (Wako); 10 pmol primer; 1.25 unit of *Taq* DNA polymerase (Wako). After this mixture was overlaid with mineral oil, it was exposed to the following temperature profile using a TR-100 thermo processor (TAITEC): one cycle of 3 min at 94℃, followed by 45 cycles of 30 sec at 94℃, 30 sec at 36℃, 2 min at 72℃ for denaturing, annealing and primer extension, respectively, and finally by one cycle of 5 min at 72℃. The amplified products were kept at 4℃ until electrophoresis.

4. Nomenclature

The amplification products were separated by electrophoresis in the 1.4% agarose gels dissolved in TAE (40 mM Tris-Acetate, 1 mM EDTA, pH 8.0), containing 1.0 μg/ml ethidium bromide. After electrophoresis, the gels were photographed under UV with Polaroid 665 film. Each amplification product was identified by its size in base pair about the primer used in the reaction. For example, OPD01-2020 refers to the 2020 base pair product amplified with primer OPD01.

In order to confirm species marker, the amplification was repeated at least twice. Only the reproducible bands in the multiple runs, regardless of their intensities, were considered in this report.

5. Data analysis

All the bands were scored as 'present' or 'absent'. Common band analysis was conducted, using pairwise comparisons among all the species to determine the genetic similarity between the each pair of species. The genetic similarities of the RAPD fragments were calculated employing the equation (Nei and Li[6]):

Similarity = $2N_{ab}/(N_a+N_b)$

Where,

$N_{ab}$ = number of shared fragments between the species 'a' and 'b'

$N_a$ = number of scored fragments of species 'a'

$N_b$ = number of scored fragments of species 'b'

The values for genetic similarity were then used for cluster analysis to generate a dendrogram.

## Results and Discussion

1. Marker identification

Fig. 1 shows the amplification products amplified using the primer OPD15 through PCR. A total of approximately 265 bands were amplified by 9 decamer primers. The number of bands for each primer varied from 4 to 16, with an average of 9 bands per primer. The size of the amplified fragments ranged from 200 bp to 3940 bp (Table 2).

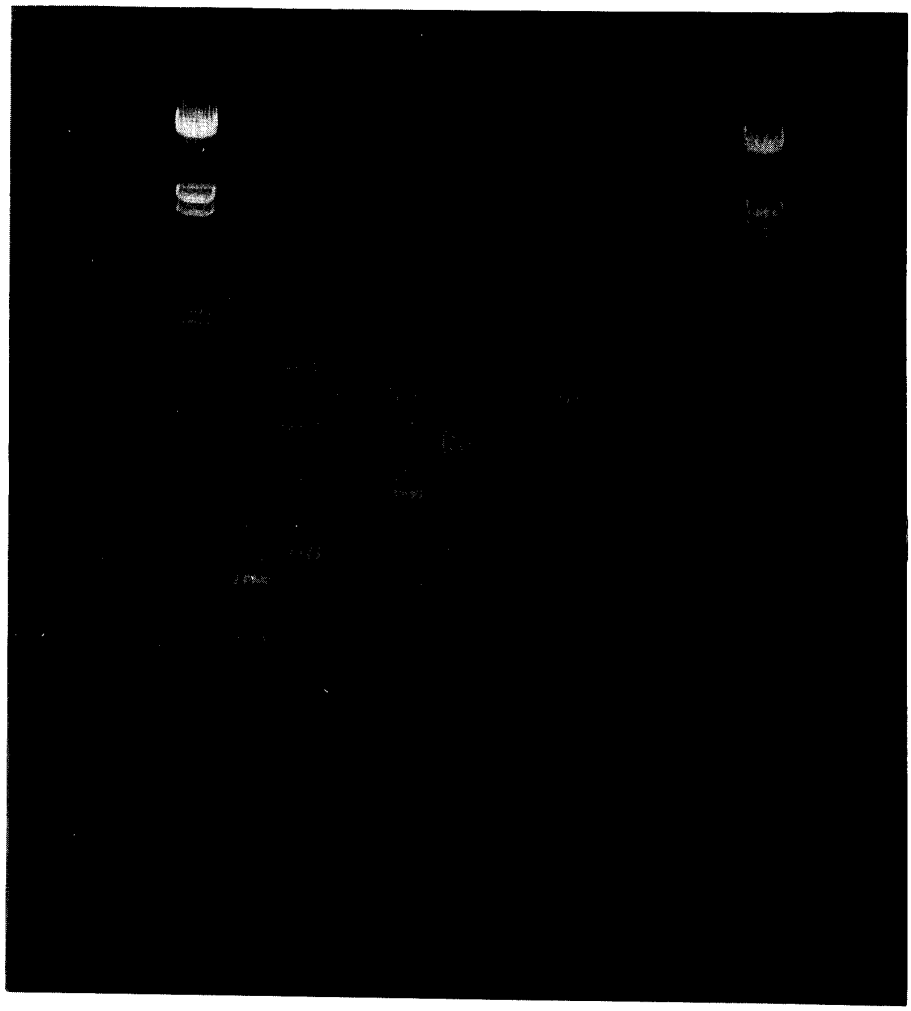

Fig. 1. Single primer PCR on total genomic DNA using arbitrary 10-mer OPD15 (5'-CATCCGTGCT). Left to right: DNA moleculer weight ( λ DNA/Hind Ⅲ and EcoR I double digest); *A. tuberosum*, *A. cowanii*, *A. caeruleum*, *A. schubertii*, *A. unifolium*, *A. albopilosum*, *A. ampeloprasum*, *A. giganteum*, *A. moly* and *A. sphaerocephalum*; DNA molecular weight ( λ DNA/Hind Ⅲ and EcoR I double digest).

Table 2. Number and size of the bands amplified by PCR with primer OPD01, OPD03, OPD05, OPD07, OPD08, OPD12, OPD15, and OPD20

| Code | Sequence | Species | | | | | | | | | | Size (bp) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | T | C | G | S | U | Al | Am | Ca | M | Sp | |
| OPD01 | 5′-ACCGCGAAGG-3′ | 5 | 7 | 8 | 13 | 9 | 8 | 5 | 6 | 8 | 11 | 340-2400 |
| OPD03 | 5′-GTCGCCGTCA-3′ | 14 | 7 | 11 | 15 | 5 | 8 | 9 | 13 | 11 | 12 | 270-2500 |
| OPD05 | 5′-TGAGCGGACA-3′ | 7 | 8 | 9 | 8 | 8 | 4 | 4 | 10 | 10 | 5 | 340-1910 |
| OPD07 | 5′-TTGGCACGGG-3′ | 8 | 9 | 12 | 4 | 8 | 12 | 8 | 10 | 11 | 8 | 290-2400 |
| OPD08 | 5′-GTGTGCCCCA-3′ | 8 | 4 | 9 | 6 | 5 | 9 | 5 | 13 | 5 | 12 | 430-1800 |
| OPD12 | 5′-GACCGTATCC-3′ | 11 | 16 | 12 | 9 | 11 | 10 | 6 | 13 | 11 | 16 | 250-2770 |
| OPD15 | 5′-CATCCGTGCT-3′ | 11 | 15 | 13 | 15 | 8 | 11 | 6 | 7 | 7 | 9 | 210-2620 |
| OPD18 | 5′-GAGAGCCAAC-3′ | 7 | 15 | 12 | 6 | 14 | 7 | 5 | 9 | 9 | 8 | 200-3940 |
| OPD20 | 5′-ACCCGGTCAC-3′ | 13 | 10 | 12 | 12 | 7 | 9 | 6 | 12 | 9 | 9 | 420-3020 |

2. Species relationships

Common bands were scored as 'present' or 'absent' and the obtaind data were used to calculate the genetic similarity between the species studied. The results are shown in Table 3. The genetic similarity scale runs from 0 (different, for all criteria studied) to 1 (identical). Values of 0.500 or less were obtained as the result of pair comparisoms made through all the species. The largest value arising between *A. albopilosum* and *A. giganteum* was 0.500. In contrast, the smallest value was 0.236 between *A. schubertii* and *A. ampeloprasum*. Cluster analyses of the genetic similarity values were conducted to generate dendrogram indicating the relationships between the respective *Allium* species studied (Fig. 2). By the way cluster analysis is a basic method for analyzing the relationships of individuals (and hence grouping them). In this dendrogram, the length of the bifurcations on the horizontal axis separating the different *Allium* species indicates their genetic similarities. The dendrogram shows that *A. albopilosum* and *A. giganteum* were the most closely related species among the examined species.

Table 3. Genetic similarities among the examined species, representing a range of relationships of *Allium*. Result based on 265 fragments amplified by 9 decamer primers

| Species | T | C | G | S | U | Al | Am | Ca | M | Sp |
|---|---|---|---|---|---|---|---|---|---|---|
| *A. tuberosum* | - | | | | | | | | | |
| *A. cowanii* | 0.457 | - | | | | | | | | |
| *A. giganteum* | 0.461 | 0.444 | - | | | | | | | |
| *A. schubertii* | 0.360 | 0.425 | 0.462 | - | | | | | | |
| *A. unifolium* | 0.327 | 0.420 | 0.358 | 0.388 | - | | | | | |
| *A. albopilosum* | 0.432 | 0.426 | 0.500 | 0.434 | 0.431 | - | | | | |
| *A. ampeloprasum* | 0.261 | 0.303 | 0.303 | 0.236 | 0.238 | 0.379 | - | | | |
| *A. caeruleum* | 0.373 | 0.413 | 0.398 | 0.354 | 0.393 | 0.374 | 0.272 | - | | |
| *A. moly* | 0.331 | 0.353 | 0.395 | 0.299 | 0.323 | 0.344 | 0.301 | 0.395 | - | |
| *A. sphaerocephalum* | 0.368 | 0.354 | 0.319 | 0.337 | 0.376 | 0.286 | 0.278 | 0.426 | 0.296 | - |

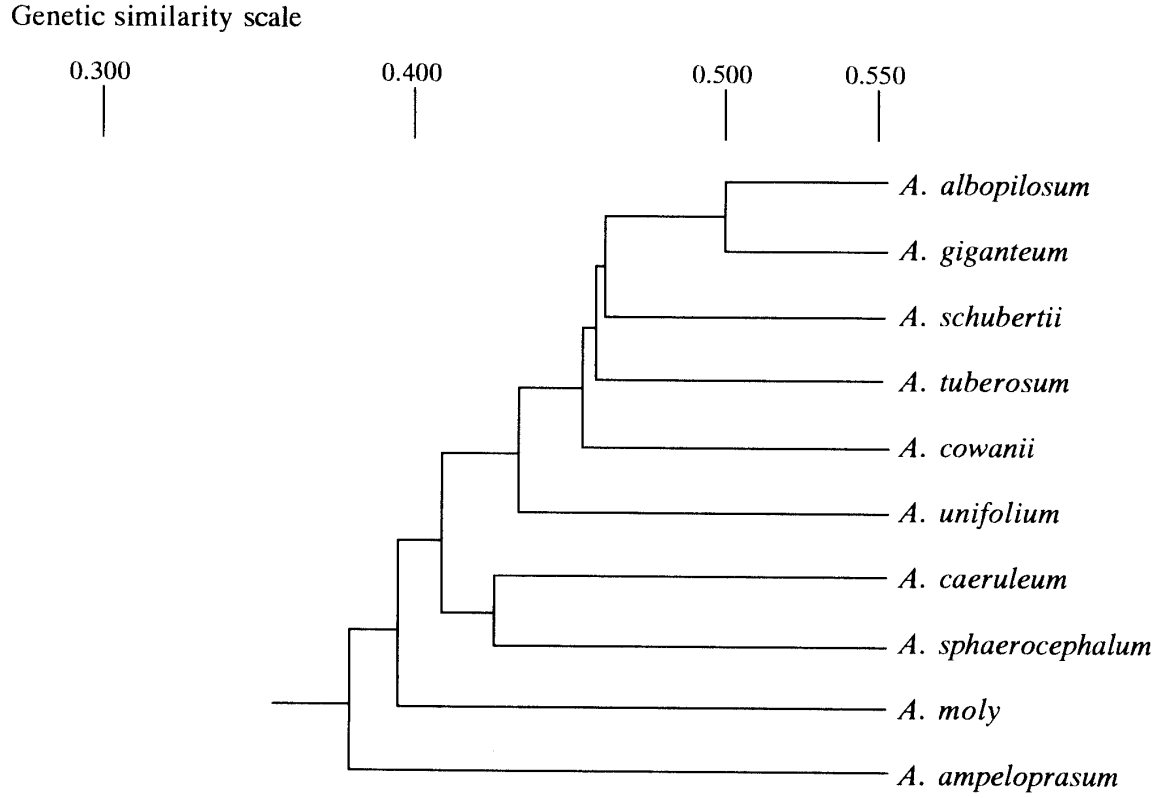


Fig. 2. Dendrogram generated by cluster analysis of genetic similarity values given in Table 3, showing relationship between the different *Allium* species.

The dendrogram based on the values of the genetic similarities of the *Alliums* shows the closer relationship between *A. albopilosum* and *A. giganteum*, followed by *A. schubertii*. This result, in general, agrees with the classification of genus *Allium* by Wendelbo[13]. According to his classification, *A. albopilosum*, *A. giganteum* and *A. schubertii* belong to section *Acanthoprason*, *Megaloprason* and *Kaloprason*, respectively; and all of them belonged to subgenus *Melanocrommyum*. *A. cowanii*, *A. tuberosum* and *A. ampeloprasum* were placed in subgenus *Molium*, *Rhizirideum*, and *Allium*, respectively. This shows some difference from Vvendersky's[10] classification. He put *A. albopilosum*, *A. giganteum*, *A. schubertii*, *A. ampeloprasum* and *A. sphaerocephalum* in the same section, *Porrum*. This dendrogram shows a more distant relationship between *A. ampeloprasum* and *A. albopilosum*.

The 'unique bands' were present in every *Allium* species. For example, OPD15-890 was only amplified in *A. tuberosum*. Some of these unique bands may be correlated to some traits specific to the species. If we shall be able to confirm the fact that the bands are really correlated to the trait, this characteristic might be used in breeding for inspecting whether the filial generation gains this trait or not. RAPD markers have been used in breeding of many other plants, such as wheat (Devos and Gale[2]) and maize (Heun and Helentjaris[4]). Though there is no evidence enough to demonstrate that these unique bands are really correlated to some traits in the present study, it may be an interesting research to examine the correlationships.

The RAPD technique has been used in many researches with its developments, such as analysis of phylogenetic relationships (Yang and Quiros[16], Tinker et al.[8]), appraisal of filial generation (Carlson et al.[1]), and so on. A large number of RAPD markers were obtained by PCR. The present study also demonstrated that an analysis of RAPD markers may be used successfully to study phylogenetic relationships among the species of *Allium*.

## Summary

RAPD analysis was applied to ten ornamental *Allium* species to assess the degrees of polymorphisms within the genus. Nine decamer primers were used, and the wide variations in banding profiles among the species were observed with every primer tested. These were assessed for use in systematic studies within the genus. Two hundred and sixty-five bands were scored (+/−) for all the species studied. Genetic similarities between each of the species were calculated and cluster analysis was used to generate a dendrogram showing phylogenetic relationships among them. The resulting analysis, in a certain degree, agreed with the previous classification of the species studied. It showed a closer relationship between *A. albopilosum* and *A. giganteum*.

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