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# Identification of Biochemical Variation in *Alternaria* Isolates of Onion Plant

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**A B S T R A C T**

Biochemical variability among the 15 isolates of *Alternaria* isolates from different locations of West Bengal was investigated in respect of isozyme to observe the polymorphism among the isolates. Electrophoretic separation of the extracts was carried out on native PAGE following the procedure and gels were stained for different enzymes. The activity of α- esterase produced distinctive bands that were dark brown, whereas the activity of β- esterase produced bands that were dark pink so that α- esterase and β-esterase were scored very easily. Two separate runs were conducted to determine reproducibility of the bands and to calculate the relative mobility (Rm). Values of each of the isozyme based on the presence or absence matrix of the bands of each isolate. Positive activity was observed for both α- and β- esterase. α- esterase enzyme showed the highest enzyme activity in terms of maximum numbers of banding loci among the two isozymes tested.

## Introduction

Onion (*Allium cepa* L.) rightly called as "queen of kitchen" is one of the oldest known and an important vegetable crop grown in India (Selvaraj, 1976). Onion is susceptible to numerous foliar, bulb and root pathogens that reduce yield and quality (Cramer, 2000). Excessive rains, humidity, temperature, pests and disease are critical factors of risk to onion cultivation. Purple blotch of onion caused by *A. porri* (Ellis) Ciff. is one of the most serious disease in India (Gupta *et al.,* 1986; Tripathi *et al.,* 2008; Ramjegathesh *et al.,* 2011). The yield losses of bulb and seed crop in India due to this disease under favourable conditions are upto 97% (Gupta and Pathak, 1998; Lakra, 1999).

As a genus, *Alternaria* is a diverse and ubiquitous group of fungi having a high degree of variability in spore shape and size, pathogenicity and sporulation and unambiguous species boundaries (Chethana *et al*, 2018). Isozyme analysis is one of the most useful methods in resolving the existence of

variation among the species. Isomer pattern of *Alternaria* spp. isoenzymes viz. α- esterase (α-Est) and β-esterase (β-Est) were studied to understand the biochemical variation among different isolates.

## Materials and Methods

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### Collection and isolation of pathogen

Leaves of onion infected by pathogen showing typical dark brown, circular to irregular spots were collected from different locations of West Bengal and fifteen *A. porri* isolates was isolated from these infected leaves by standard tissue isolation technique in the laboratory. The infected leaf bits will be surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 30 seconds and repeatedly washed separately in sterilized distilled water to remove the traces of mercury if any and then transferred to sterilized Petri plates (1-2 leaf bits per Petri dish) containing potato dextrose agar (PDA). The Petri plates will be incubated at room temperature (27±1°C) and observed periodically for the growth. Bit of fungal growth developed from the infected tissue was transferred to PDA slants. Then the mycelial tip or single spore isolation will be done for purification of the pathogen. Then such slants with pure culture will be used for further studies.

### Biochemical variability

### Studies on isoenzyme profiles of *Alternaria* isolates by polyacrylamide gel electrophoresis method

Isozyme analysis is one of the most useful methods in resolving the existence of variation among the species. Isomer pattern of *Alternaria* spp. isoenzymes viz. α- esterase (α-Est) and β-esterase (β-Est) were studied to understand the biochemical variation among different isolates. Electrophoresis of esterase isoenzyme was done in 7.5% gel according to the method proposed by Kahler and Allard (1970).

### Preparation of buffer solution

a) Buffer solution A

| | |
|---|---|
| Tris (Hydroxy methyl amino methane) | 0.8g |
| Citric acid | 200mg |
| Double distilled water | 100 ml |
| pH was adjusted to | 7.8 |

b) Tank electrode buffer (B)

| | |
|---|---|
| Lithium hydroxide | 1.2gm |
| Boric acid | 11.9gm |
| Double distilled water | 1000ml |
| Adjusted to pH | 8.2 |

c) Buffer C

| | |
|---|---|
| Tris (Hydroxy methyl amino methane) | 310mg |
| Citric acid | 80mg |
| Double distilled water | 1000ml |
| Adjusted to pH | 8.2 |

d) Buffer D

| | |
|---|---|
| Tris (Hydroxy methyl amino methane) | 600 mg |
| Double distilled water | 100 ml |

e) Gel casting solution (7.5 % gel)

| | |
|---|---|
| Acrylamide | 3.25 gm |
| Bis-acrylamide | 0.067 gm |
| Double distilled water | 50 ml |

f) Amonium persulphate solution (APS)

| | |
|---|---|
| Amonium persulphate solution (APS) | 250mg |
| Double distilled water | 5 ml |
| TEMED (N,N,N,N(Tetramethyl ethylene diamine) | 5µl |

This solution was freshly prepared on the day of use

g) Loading buffer

| | |
|---|---|
| Bromophenol blue (5 %) and Glycerol | 1: 2 |

## Preparation of staining solution

| | |
|---|---|
| Fast blue R R salt | 100 mg |
| α or β-naphthyl acetate | 0.004 g |
| Buffer (D) | 100 ml |

## Extraction of enzyme from isolates of *Alternaria* spp.

Enzymes were extracted individually from different isolates. 500 mg fungal mat freshly harvested from actively growing culture of *Alternaria* isolates were crushed with 1ml enzyme extraction buffer (pH-7) in a pre-cooled mortar and pestle at $^0$C. The resulting homogenate mixture was centrifuged at 14,000 r.p.m for 20 mins at 4ºC in Heraeus Biofuge (Stratos, Biorad), a temperature controlled centrifuge machine. The supernatant was collected and kept in the refrigerator at 4ºC and used as enzyme source.

## Gel casting

7.5% (size 10cm x10cm micro) gel was prepared by mixing 27 ml buffer (B) and 3 ml buffer (C with 3.25 g of acrylamide, 0.067 g bis-acrylamide. After thorough mixing, 5 ml of ammonium persulphate (APS) solution and 20µl TEMED (N, N, N', N'-tetramethylethylene diamine) was added to it. Then quickly pour the gel solution in the gap between the two glass plates and set the comb by inserting it between the glasses and left it to solidify. After solidification, the samples of different isolates were loaded separately in different lanes and the gel was run at 80 volt for 2.5 – 3 h 4$^0$C.

## Staining of gel

a) α esterase gel

On completion of the gel run, the gel was carefully removed from and placed into the staining solution (100 ml of buffer D) prepared at the time of use containing 100mg Fast Blue RR salt and 0.004 g α-napthyl acetate(dissolved in 1 ml of ethyl alcohol). The gel was incubated at 28 ºC in dark condition for 30 min with occasional shaking for development of band. After development of the bands, the gel was washed with distilled water. The gel was transferred and photographed. The Rm (Relative mobility) values of band(s) were estimated. The banding patterns or the zymograms so obtained were analyzed based on procedure given for identifying the putative loci as described by Wendem and Weeden (1989).The band length was measured and relative mobility (Rm) value was calculated using the following formula.

$$\text{Rm value} = \frac{\text{Distance of the band from origin}}{\text{Distance of buffer front}}$$

b) β-esterase gel

On completion of the gel run, the gel was carefully separated and placed in 100 ml of buffer (D), containing 100mg Fast Blue RR salt and 0.004g β-napthyl acetate (dissolved in 1 ml of ethyl alcohol) for staining. The gel was incubated at 28 $^0$C in dark condition for 30 min with occasional shaking for development of band. After development of the bands, the gel was washed with distilled water. The gel was transferred and photographed. The Rm (Relative mobility) values of band(s) were estimated as mentioned before.

## Results and Discussion

Biochemical variability among the 15 isolates of *Alternaria* isolates from different locations was also investigated in respect of isozyme to observe the polymorphism among the isolates and the results are discussed below.

## Alpha esterase isozyme

Electrophoretic separation of enzymes, which exploits the polymorphism of detected isozyme forms, is an important biochemical/ molecular technique that have been widely used to generate a large number of markers for the assessment of genetic diversity in fungi. An experiment was conducted to study the biochemical variability among *Alternaria* isolates collected from different locations based on α-esterase profiling.

Electrophoretic separation of the extracts was carried out on native PAGE following the procedure described by Davis (1964) and gels were stained for different enzymes. The activity of α- esterase produced distinctive bands that were dark brown, whereas the activity of β-esterase produced bands that were dark pink so that α- esterase and β- esterase were scored very easily. Two separate runs were conducted to determine reproducibility of the bands and to calculate the relative mobility (Rm). Values of each of the isozyme based on the presence or absence matrix of the bands of each isolate. Positive activity was observed for both α- and β- esterase. α- esterase enzyme showed the highest enzyme activity in terms of maximum numbers of banding loci among the two isozyme tested. All the isolates have different banding pattern and maximum locii 7 was observed on isolate $AP_8$. Five isolate produced 6 banding patterns ($AP_1$, $AP_2$, $AP_4$, $AP_7$ and $AP_{15}$) and another five isolate produced 5 banding patterns ($AP_5$, $AP_{11}$, $AP_{12}$, $AP_{13}$ and $AP_{14}$). Four isolate produced 4 banding patterns ($AP_3$, $AP_6$, $AP_9$ and $AP_{10}$). It was also observed that all the 15 isolates have one loci of Rm value 0.31. Among the 15 isolates, 12 isolates produced one loci on the Rm value of 0.29 except $AP_5$, $AP_6$ and $AP_4$. Similarly all the isolates produced another loci of Rm value 0.32except isolate $AP_5$, $AP_6$, $AP_2$ and $AP_1$. Among the 15 isolates, 9 isolates also produced another loci of Rm value of 0.33 except $AP_9$, $AP_{10}$, $AP_{15}$, $AP_7$, $AP_3$, $AP_2$ and $AP_1$. Similarly the 9 isolates of among 15 isolates, also produced another loci of Rm value 0.36 except $AP_{11}$, $AP_{12}$, $AP_{13}$, $AP_{14}$, $AP_6$ and $AP_8$. The two isolates $AP_5$ and $AP_{15}$ also produced one loci of Rm value 0.40. 6 isolates ($AP_{15}$, $AP_7$, $AP_8$, $AP_4$, $AP_2$ and $AP_1$produced another bands on Rm value of 0.41. Isolates $AP_6$, $AP_8$, $AP_2$ and $AP_1$ produced another Rm value of 0.42. The highest Rm value 0.51 was observed on 7 isolates were $AP_5$, $AP_6$, $AP_7$, $AP_8$, $AP_4$, $AP_2$ and $AP_1$. This indicated that these 15 isolates were different in isozyme pattern of α- esterase (Fig. 1).

A dendrogram was generated by UPGMA clustering as presented in the Figure 2. This dendrogram identified three major clusters with 25% euclidean distance. One cluster (group I) comprised of seven isolates $AP_{14}$, $AP_{15}$, $AP_{10}$, $AP_1$, $AP_2$, $AP_{13}$ and $AP_7$ while other cluster (group II) comprised of four isolates $AP_8$, $AP_{12}$, $AP_9$ and $AP_{11}$ and another cluster (group III) comprised of four isolates $AP_5$, $AP_6$, $AP_3$ and $AP_4$. Group I was further sub-clustered into two groups, of which first sub-cluster (group IA) had three isolates $AP_{14}$, $AP_{15}$ and $AP_{10}$ in which $AP_{14}$ and $AP_{15}$ showed their close relationship and isolate $AP_{10}$ was in separate individual cluster. Second sub-cluster (group IB) included four isolates $AP_1$, $AP_2$, $AP_{13}$ and $AP_7$. Group IB was again sub divided into two clusters, i,e group IBa and group IBb. Group IBa comprised of three isolates $AP_1$, $AP_2$ and $AP_{13}$ and group IBb had only one isolate $AP_7$. Isolate $AP_{13}$ was in separate individual cluster while isolates $AP_1$ and $AP_2$ shared very close relationship. Group II was belongs to sub-clustered into two, of which first sub-cluster (group IIA) had two isolates $AP_8$ and $AP_{12}$ which are closely related while the second sub-cluster (group IIB) had also two isolates $AP_9$ and $AP_{11}$ which are also closely related. Group III was comprised of four isolates $AP_5$, $AP_6$, $AP_3$ and $AP_4$ which were closely related with each other.

**Fig.1** Zymogram of Alpha esterase isozyme of *Alternaria* isolates

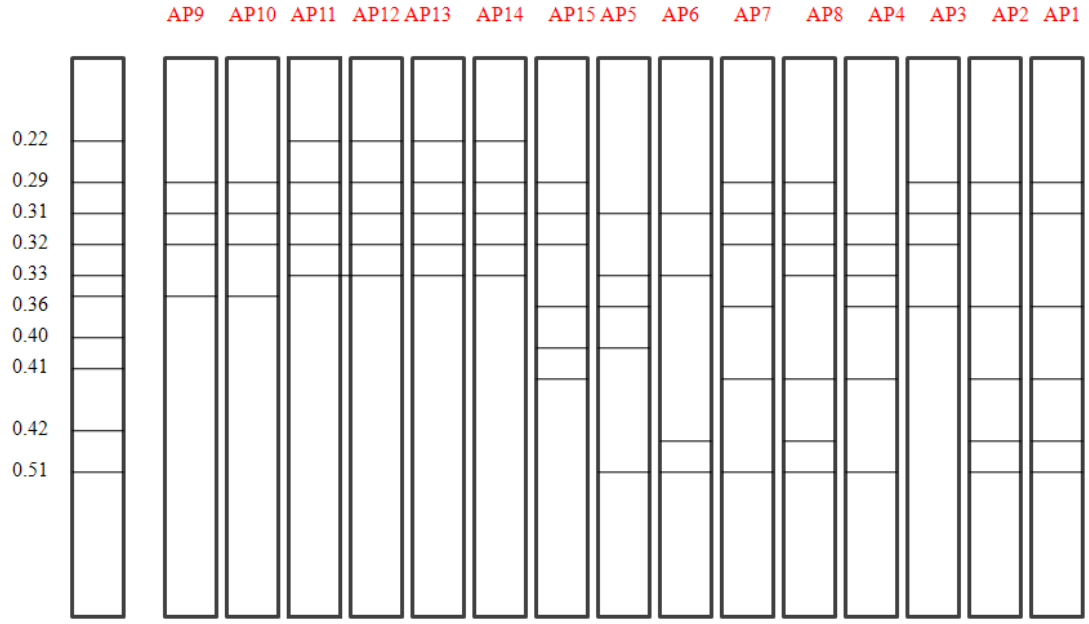
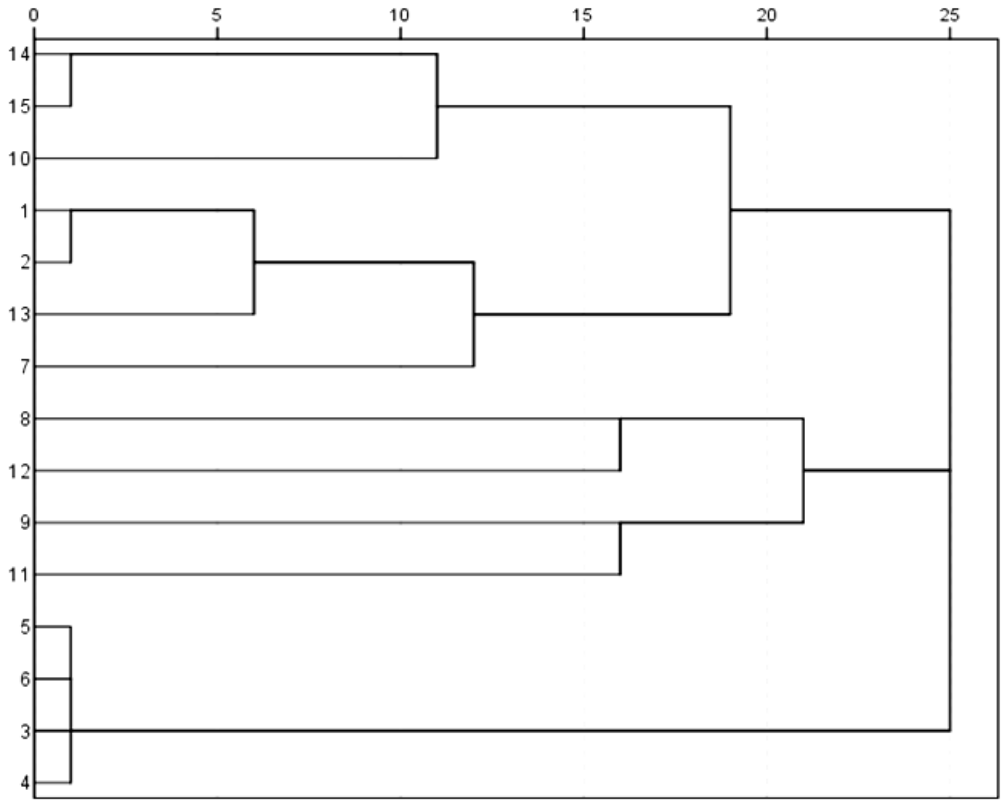



**Fig.2** Dendrogram for Alpha esterase isozyme data, showing relationships among *Alternaria spp*




Dendrogram using Avarage Linkage (Between Groups) Rescaled distance cluster combine

**Fig.3** Zymogram of Beta esterase isozyme of *Alternaria* isolates

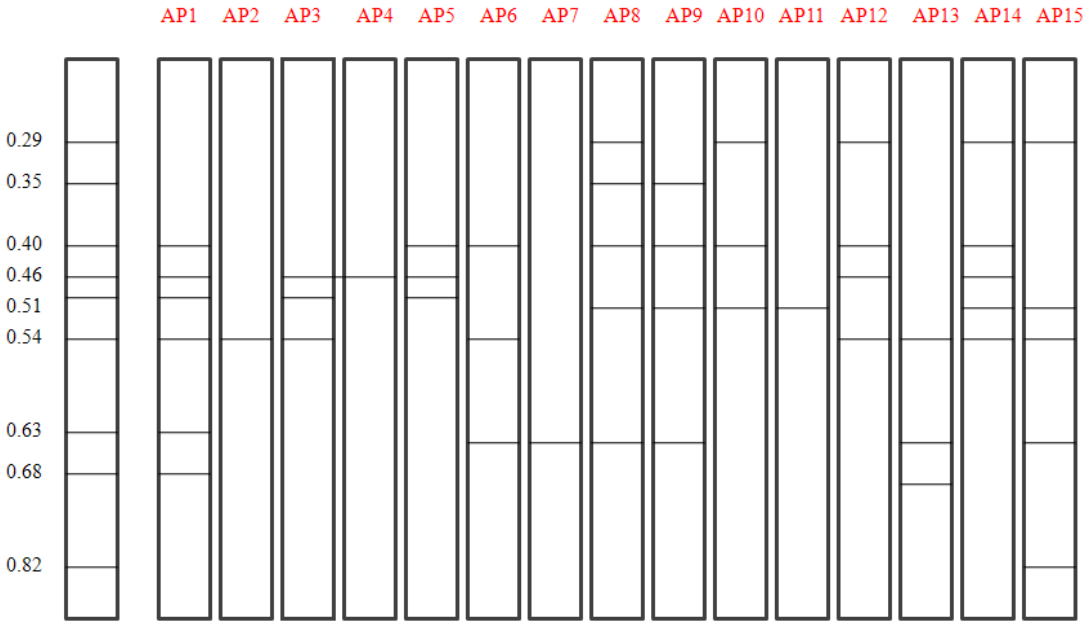
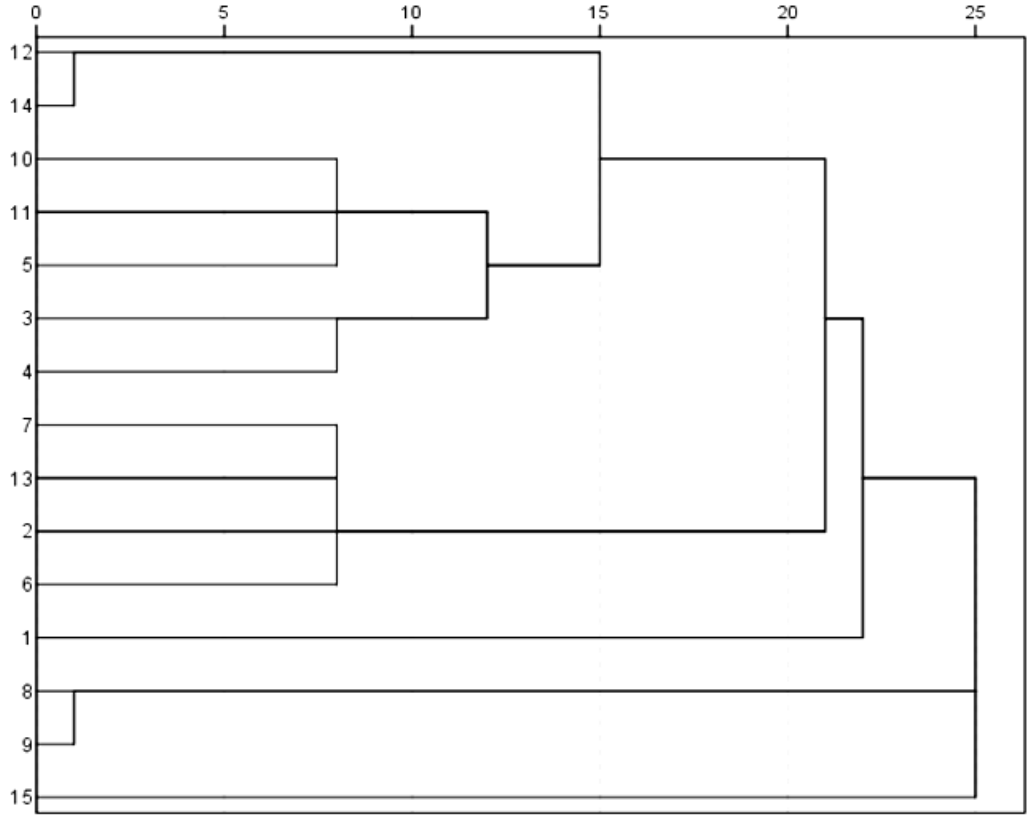



**Fig.4** Dendrogram for beta esterase isozyme data, showing relationships among *Alternaria spp*



## Beta esterase isozyme

A separate experiment was conducted to study the biochemical variability among *Alternaria* isolates collected from different locations based on ß-esterase profiling.

All the isolates have different banding pattern and maximum locii 6 was observed on isolate $AP_1$. Three isolate produced 5 banding patterns ($AP_8$, $AP_{14}$ and $AP_{15}$) and another five isolate produced 3 banding patterns ($AP_3$, $AP_5$, $AP_6$, $AP_{10}$ and $AP_{13}$). Two isolates produced 4 banding patterns ($AP_9$ and $AP_{12}$) and four isolates produced 1 banding patterns ($AP_2$, $AP_4$, $AP_7$ and $AP_{11}$). Among the 15 isolates, isolate $AP_{15}$ produced one loci of highest Rm value of 0.82. Five isolates ($AP_8$, $AP_{10}$, $AP_{12,}$ $AP_{14}$ and $AP_{15)}$ produced another loci of Rm value 0.29 and two isolates ($AP_8$ and $AP_{9)}$ produced another Rm value of 0.35. Among the 15 isolates, 8 isolates also produced another loci of Rm value of 0.40 except $AP_2$, $AP_3$, $AP_4$, $AP_7$, $AP_{11}$, $AP_{13}$ and $AP_{15}$. Similarly the 9 isolates of among 15 isolates, also produced another loci of Rm value 0.51 except $AP_2$, $AP_4$, $AP_{6,}$ $AP_7$, $AP_{12}$ and $AP_{13.}$ 8 isolates also produced another loci of Rm value of 0.54 except $AP_4$, $AP_5$, $AP_7$, $AP_8$, $AP_9$, $AP_{10}$ and $AP_{11}$. Similarly the 7 isolates ($AP_1$, $AP_6$, $AP_7$, $AP_8$, $AP_{9,}$ $AP_{13}$ and $AP_{15}$) of among 15 isolates, also produced another loci of Rm value 0.63 and 6 isolates ($AP_1$, $AP_3$, $AP_4$, $AP_5$, $AP_{12}$ and $AP_{14}$) produced another bands on Rm value of 0.46. The two isolates $AP_1$ and $AP_{13}$ also produced one loci of Rm value 0.68. This indicated that these 15 isolates were different in isozyme pattern of ß-esterase (Fig. 3).

A dendogram was generated by UPGMA clustering as presented in the Figure 4. This dendrogram identified three major clusters with 25% euclidean distance. One cluster (group I) comprised of twelve isolates $AP_{12}$, $AP_{14}$, $AP_{10}$, $AP_{11}$, $AP_5$, $AP_3$ $AP_4$, $AP_7$, $AP_{13}$, $AP_2$, $AP_6$ and $AP_1$ while other cluster (group II) comprised of two isolates $AP_8$ and $AP_9$ and another cluster (group III) comprised of only one isolate $AP_{15.}$ Group I was further sub-clustered into two groups, of which first sub-cluster (group IA) had eleven isolates and was again sub divided into two clusters, i,e group IAa and group IAb. Group IAa comprised of seven isolates $AP_{12}$, $AP_{14}$, $AP_{10}$, $AP_{11}$, $AP_5$, $AP_3$ and $AP_4$ while isolates $AP_{12}$ and $AP_{14}$ shared very close relationship. Group IAb had four isolate $AP_7$, $AP_{13}$, $AP_2$ and $AP_6$ which were closely related with each other. Second sub-cluster (group IB) included only one isolate $AP_1$ and was in separate individual cluster. Group II was comprised of two isolates $AP_8$ and $AP_9$ which were closely related with each other. Group III was comprised of only one isolate $AP_{15}$ and was in separate individual cluster.

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