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Comparative Morphology, Genetic Variability and Taxonomy of Genus *Phoma* and its Agriculturally Important Species

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The anamorphic genus *Phoma* includes many important plant pathogens. The classification of the *Phoma* species based on morphology is very difficult as they reveal multiple "species-specific" characters. Therefore an attempt has been made to describe taxonomical key and illustrate the cultural and morphological charcters *ie.* Growth and pigmentation of cultures, shape and size of pycnidia, condia, chlamydospores and sclerotia, of fourteen agriculturally important species of Genus *Phoma* to provide an authentic identification protocol to the end users. The genetic relatedness among the twenty seven isolates representing fourteen *Phoma* species was also evaluated based on Randomly amplified polymorphic DNA (RAPD) and Inter simple sequence repeat (ISSR) markers. RAPD showed more polymorphism within particular *Phoma* species by seprating them into three clusters, whereas based on ISSR markers, isolates were grouped into five clusters and showed more polymorphism within different *Phoma* species. ISSR primers were also able to separate the *Phoma* species on the basis their section *viz.* Peyronellaea, Phoma, Phyllostictoides and Pilosa. Only one species, *P. gardeniae* belongs to section Paraphoma was not cluster separately. Overall, ISSR markers were found a much more efficient tool to differentiate variability among the different isolates of *Phoma* species.

Key words: Morphology, *Phoma* species, RAPD, ISSR, Genetic diversity.

Fungal genus *Phoma*[1] has been reported as phytopathogens, saprophytes from soils, aquatic and aerial environment, marine environment, entomopathogenic and as opportunistic human pathogens[47]. More than 2000 species have been studied[43] .The major objective of any taxonomic study includes systematic grouping of taxa of interest through the generation of robust natural classification based on constant characterststics, which reveal their factual evolutionary record and development of trustworthy identification key(s) for uncomplicated taxon determination[68] .

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According to Saccardo's taxonomic system, the genus *Phoma* refers to 'pycnidia with one celled hyaline conidia occurring on herbaceous stems'[67]. Boerema and his colleagues have contributed significantly to the reclassification and numerous other aspects of *Phoma* and *Phoma*-like fungi[11, 33]. An extensive study of *Phoma* taxonomy was recently published as *Phoma* Identification Manual based on morphological charcters[33]. But the classification of *Phoma species* is still controversial. Confusing and overlapping characters are reported among several *Phoma* species within same sections. Few studies were also done on cultural and morphological charcterization of Indian species of *Phoma*[53, 65]. But there is no extensive studies have been done on development of identification key(s) for taxon

determination of agriculturally important *Phoma* species.

Variability, taxonomic and evolutionary studies in microorganisms have increased with the development of molecular techniques. Randomly amplified polymorphic DNA (RAPD) is an effective molecular fingerprinting tool that identifies genetic variation among populations[69]. Inter simple sequence repeat (ISSR) markers are powerful tools to reveal the variation in genome microsatellite regions[44]. On the basis of such studies, it could be said that the knowledge of the genetic structure of pathogen populations has direct agricultural applications. For instance, the genetic variation maintained within a population indicates the speed at which a pathogen evolves[42]. Therefore, these molecular tools presumably would be useful to determine the genetic variability in the different isolates of *Phoma* spp.

In the light of above facts, This study was conducted to revise the taxonomy of fourteen agriculturally important *Phoma* species and to evaluate the genetic variation occurring among different isolates of *Phoma* spp. with different geographic origins based on RAPD and ISSR markers.

MATERIALS AND METHODS

Collection of *Phoma* isolates

Phoma[31] isolates were taken from Indian Type Culture Collection (ITCC), Division of Plant Pathology, IARI, and New Delhi. The collected isolates were from different places and sources. The same isolates were used for the genetic diversity analysis studies based on RAPD and ISSR markers. Details of the accession number, name of the species, source and place of both *Phoma* species isolates were given in Table 1.

Morphological characterization:

The cultures of *Phoma* isolates were grown on Oat Meal Agar medium in Petri dish at 28±2 °C and examined at different stage of growth for 30 days. The isolates were caterorized in to different groups based on the different parameters *viz.*, Growth and pigmentation of cultures, shape and size of pycnidia, condia, chlamydospores and sclerotia.

Genomic DNA Extraction

Genomic DNA was extracted from frozen mycelium of *Phoma* spp. based on Cetyltrimethyl ammonium bromide (CTAB) mini extraction method[35]. The DNA concentration and purity of the samples was determined with Nano Drop Spectrophotometer (Thermo Scientific).

Variability among the different isolates of *Phoma* spp. using different RAPD Markers

DNA from 27 isolates was subjected to genetic diversity analysis by the RAPD method using 10 randomly chosen 10-base random primers. Among them six primers were chosen for further studies (Table 2). PCR reactions were carried out in 0.2 ml thin-walled PCR tubes with a total reaction volume of 25 µl containing 2.5 µl of 10X buffer, 2.5mM of each dNTPs(0.5µl) ,1µl of primer, 0.5 µl $MgCl_2$ (25mM) and 1U (0.2 µl) of Taq polymerase (Bangalore, Genei). The PCR amplification conditions were initial denaturation at 94°C for 3 min followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 35 °C for 1 min, primer extension at 72°C for 2 min, and a final primer extension at 72°C for 5 min. Six primers of RAPD were selected for final analysis based on informative banding patterns, clarity, and repeatability.

Variability among the different isolates of *Phoma* spp. using different ISSR Markers

ISSR analysis was carried out amplifying the genomic DNA using six ISSR primers. Among them five primers were chosen for further studies (Table 3).The PCR amplification conditions were: Initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 1 min, primer annealing at 34 °C for 1 min, primer extension at 72°C for 1 min, and a final primer extension at 72°C for 5 min. A total of six primers were screened initially. Five primers were selected for final analysis based on informative banding patterns, clarity, and repeatability.

Scoring the amplified fragments

The amplified products along with 1X loading dye were separated on 1.2 per cent agarose gel at 70 volts (45 volts per cm of gel) using 1 x TAE buffer of pH 8.0 containing ethidium bromide (0.50 µg/ml). The amplified profiles for all the primers were compared with each other and the bands of DNA fragments were scored as 1 for the presence and 0 for the absence of a band generating the '0', '1' matrices. The gels were photographed using gel documentation system.

Statistical analysis

DNA bands that could be scored unequivocally for presence or absence were included in the analysis. Faint bands were also scored. Binary matrices were analyzed by NTSYS-PC (version 2.0; Exeter Biological Software, Setauket, NY). Jacard's coefficients were clustered to generate dendrogram using the SAHN clustering programme, selecting the unweighted pair group method with arithmetic average (UPGMA) algorithm in NTSYS-PC[66].

RESULTS

Comparison of morphological traits for species differentiation

1. Conidia formed from ampulliform to doliiform phialides……2
2. Cultures producing either chlamydospores or sclerotia……3
2. Cultures not producing accessory vegetative or spore states……5
3. Chlamydospores multicellular, alternariod ……4
4. Colonies consistently with salmon pink patches; conidia 4-5X2-2.5 µ…..……*Phoma sorghina*
4. Chlamydospores catenate; conidia 5-9X 2.5-3 µ……..*P. glomerata*
5. Pycnidia lacking setae, not rostrate ……6
5. Pycnidia lacking setae, rostrate, cultures variable, occasionally with unicellular chlamydospores; conidia 5-6X2-2.5 µ……*P. multirostrata*
6. Colonies with vinaceous to red pigmentation ……7
6. Colonies lacking such pigmentation ……8
7. Aerial mycelium sparse, reverse vinaceous; conidia 3.5-5X2 µ, biguttulate…….*P. destructiva*
7. Aerial mycelium sparse, reverse red, turning blue with NaOH; conidia 4-5X1.5-2 µ…… .....*P. herbarum*
8. Conidia often becoming 1 septate ……9
8. Conidia unicellular …… ……10
9. Colonies scalloped or lobed at margin, NaOH turns agar blue-green; conidia 5.5-10X2.5-3.5 µ, often becoming 1 septate…… ……..……*P. exigua*
9. Colonies olivaceous grey, not scalloped, stilboid tufts present, pycnidia sparse; conidia 4.5-9X2.5-3.5 µ, becoming 1 septate…… ……*P. lycopersici*
9. Colonies colourless/dull green to olivaceous/ olivaceous grey, reverse buff to dull green/ olivaceous to leaden grey leaden black, aseptate conidia 4–8X 2–3 µ, septate conidia up to 10X4.5 µ……*P. cucurbitacearum*
10. Conidia width not exceeding 1.5 µ…… ……11
10. Condia width >2 µ…… ……12
11. Colonies floccose, reverse olivaceous to black; pycnidia pseudoparenchlymatous; conidia 3-4X1.5 µ, irregular gullulate…… ……*P. tropica*
11. Colonies variable; pycnidia pseudoparenchlymatous to pseudosclerenchlymatous, often sterile and then sclerotia; conidia 3.5-4.5X1.5 µ……..……*P. lingum*
12. Conidia l/b ration <2/ 1……13
12. Conidia l/b ratio >2/ 1……14
13. Colonies with dense to moderately dense aerial mycelium; reverse yellow-saffron; conidia 3-3.5X2-2.5 µ, often with single guttule…… …….*P. fimeti*
13. Conidia 3–4X2–2.5 µ, with mostly 2 or 3 conspicuous greenish guttules. Colonies producing a yellow pigment that stains the agar (reverse) honey to umber.....*P. putaminum*
14. Colonies olivaceous brown; pycnidia abundant in area lacking aerial mycelium; conidia 6-9X3.5-4.5 µ, often with single guttule…… ……..*P. betae*
14. Colonies with distinct apricot or scarlet tinges on OA, with NaOH spot test quickly changing to purplish/blue. Conidia mostly 3.5–5X1.5–2.5 µ, sometimes with a few guttules .....*P. multipora*

14. Growth rate fast, 5–7 cm on OA after 7 days, conidia highly variable and relatively large, 3.5–10.5X1.5–4.5 µ, colony greenish on OA .....*P. gardeniae*

Taxonomy description of the genus *Phoma*

Mycelium immersed, branched, septate, hyaline or pale brown. Conidiomata pycnidial, immersed, or semi-immersed, sometimes becoming erupent, unilocular, brown, globose, separate or aggregated, occasionally confluent, thin walled; walls of thin walles, pale to medium brown texture angularis. Ostioles single or several to each pycnidium, central, not papillate. Conidigenos cells enteroblastic, phialidic, integrated or discrete, ampulliform to doliiform, hyaline, smooth, collarette and aperture minute, periclinal wall markedly

Table 1. Details of isolates of *Phoma* species used in present study

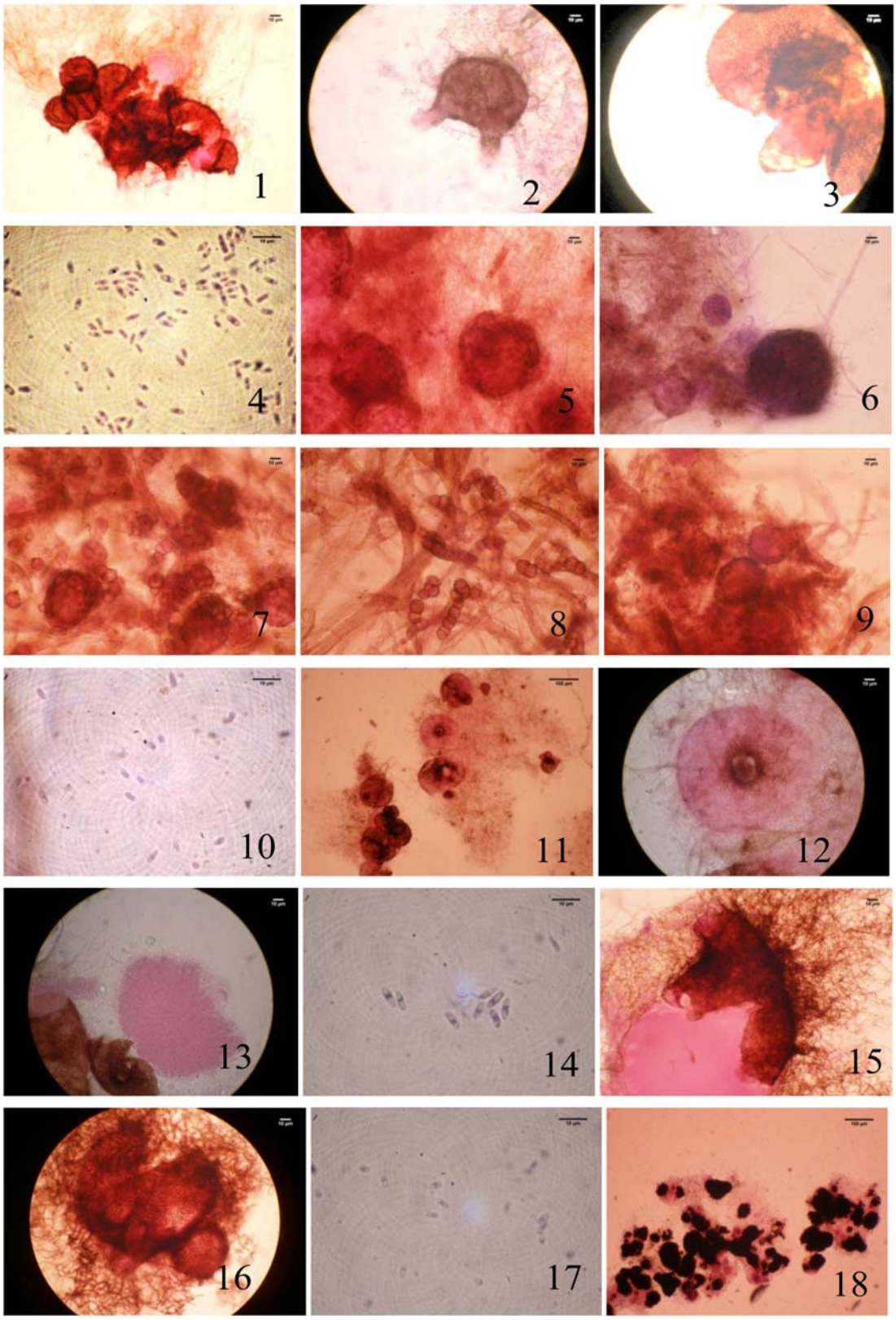
S.No	ITCC No.	*Phoma* species	Source	Place	Section
1	4554	*P. gardeniae*	*Gardenia resinifera*	Junagarh	Paraphoma
2	3712	*P. glomerata*	*Ficus religiosa*	New Delhi	Peyronellaea
3	6236	*P. glomerata*	*Ficus religiosa*	Waghapur	Peyronellaea
4	6949	*P. glomerata*	Jamun tree	Jobner	Peyronellaea
5	2428	*P. sorghina*	-	Hyderabad	Peyronellaea
6	3176	*P. sorghina*	Brinjal	Shimla	Peyronellaea
7	4160	*P. sorghina*	Paddy	Bhopal	Peyronellaea
8	2086	*P. capitulum*	Soil	New Delhi	Phoma
9	6235	*P. destructiva*	Soil	Pune	Phoma
10	2088	*P. fimeti*	Soil	New Delhi	Phoma
11	2090	*P. multipora*	Soil	New Delhi	Phoma
12	2251	*P. multirostrata*	Soil	Jodhpur	Phoma
13	6031	*P. multirostrata*	*Catharanthus roseus*	Shimoga	Phoma
14	2164	*P. putaminum*	Soil	Assam	Phoma
15	2258	*P. tropica*	Brinjal	New Delhi	Phoma
16	2811	*P. tropica*	Brinjal phyllosphere	Ujjain	Phoma
17	3765	*P. tropica*	Kulthi	New Delhi	Phoma
18	3796	*P. tropica*	*Momordica charantia*	Warangal	Phoma
19	3767	*P. tropica*	Soybean	New Delhi	Phoma
20	6206	*P. tropica*	*Dolichous lablab*	Navsari	Phoma
21	2087	*P. cucurbitacearum*	Soil	New Delhi	Phyllostictoides
22	2049	*P. exigua*	Soil	New Delhi	Phyllostictoides
23	2478	*P. exigua*	Soil	Hyderabad	Phyllostictoides
24	3601	*P. exigua*	Tannery effluent	Agra	Phyllostictoides
25	5546	*P. exigua*	Large Cardamom	Kalimpong	Phyllostictoides
26	6962	*P. lycopersici*	Tomato leaf	NCIPM	Phyllostictoides
27	2238	*P. betae*	*Beta vulgaris seed*	New Delhi	Pilosa

Table 2: Sequences of random primers used in RAPD analysis for different isolates of *Phoma* species

Primer	Sequence 5' – 3'
OPA-11	CAATCGCCGT
OPN-20	GGTGCTCCGT
OPE- 14	TGCGGCTGAG
OPA 3	AGTCAGCCAC
OPA-2	TGCCGAGCTG
OPB-13	CTGGCGAACT
OPB-11	TGGGGGACTC
OPO-4	AAGTCCGCTC

Table 3: Sequences of primers used in ISSR analysis for different isolates *Phoma* species

Primer	Sequence 5' – 3'
ISSR-1	(GAGA)4 GAT
ISSR-2	ACTGACTGACTGACTG
ISSR-3	(GAGA)4 GAAT
ISSR-4	(ACC)4Y
ISSR-5	GACACGACACGACACGACAC
ISSR-6	(GATA)4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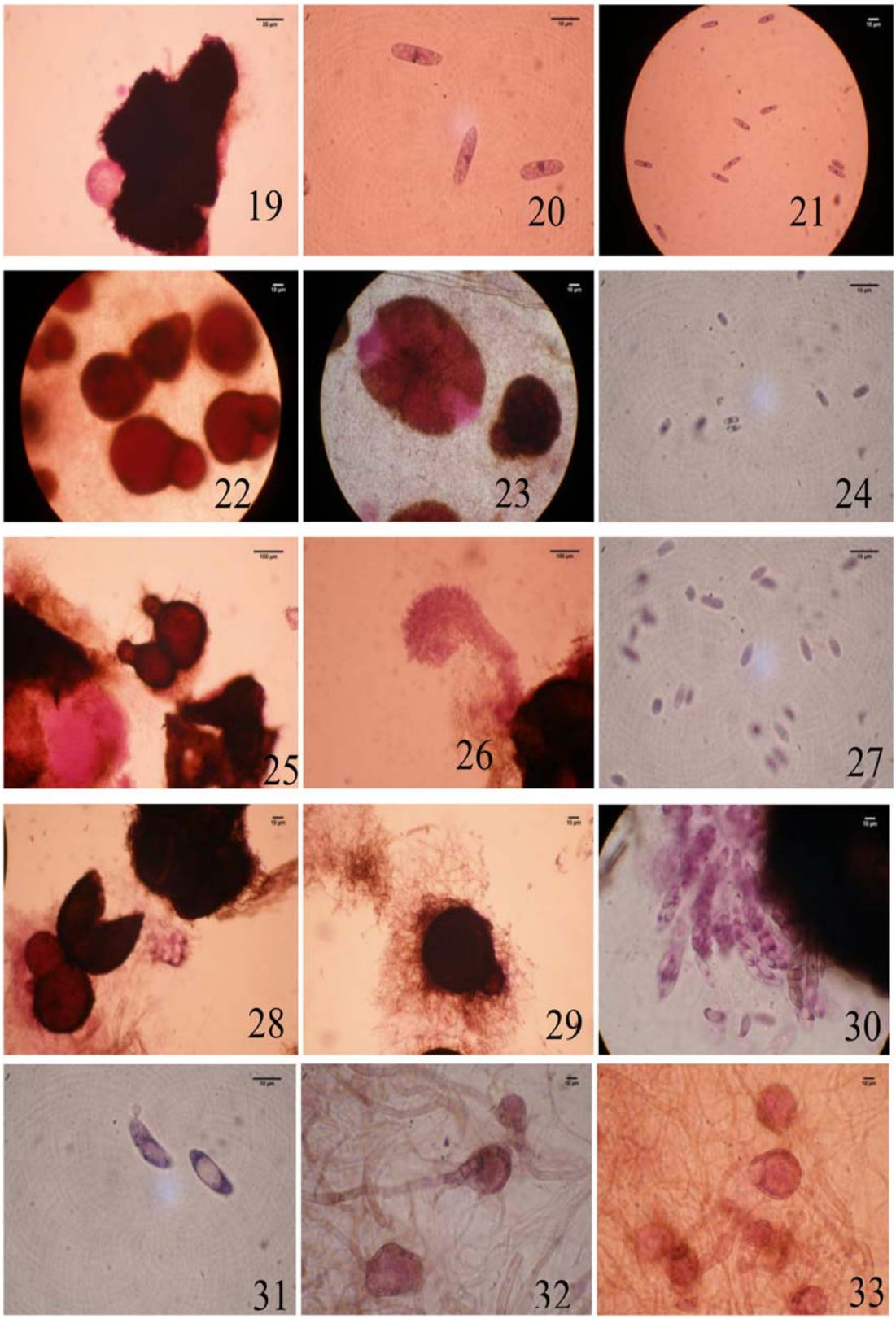
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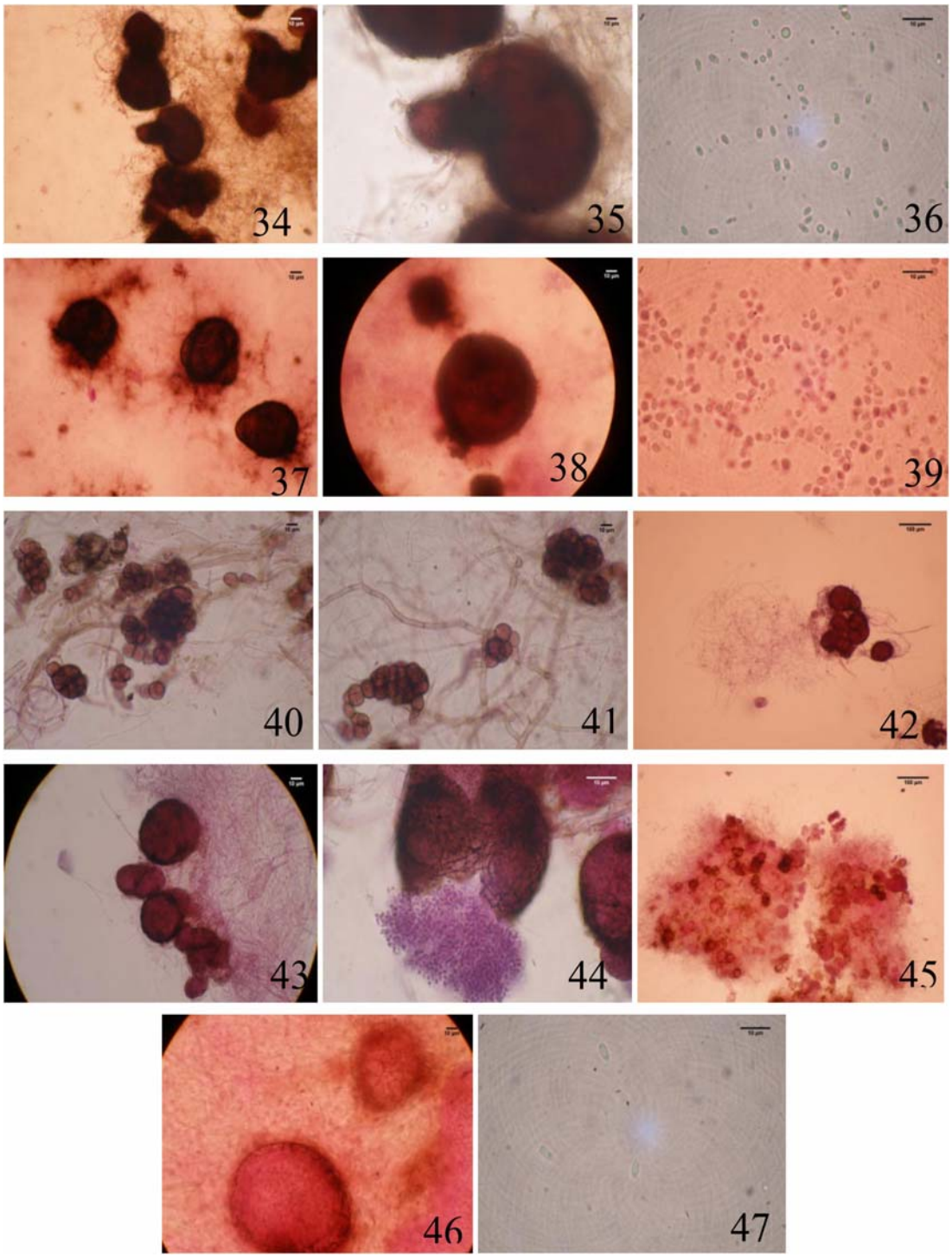

Fig. 1. (1-4) *Phoma betae,* (5-6) *Phoma capitulum,* (7-10) *Phoma cucurbitacearum,* (11-14) *Phoma destructiva,* (15-17) *Phoma exigua,* (18-21) *Phoma fimeti,* (22-24) *Phoma gardeniae,* (25-27) *Phoma glomerata,* (28-31) *Phoma lycopersici,* (32-33) *Phoma multipora,* (34-36) *Phoma multirostrata,* (37-39) *Phoma putaminum,* (40-44) *Phoma sorghina,* (45-47) *Phoma tropica*

thicker. Conidia hyaline, aseptate or occasionally 1 septate, thin walled, often guttulate, ellipsoid, cylindrical, fusiform, pyriform or globose.

Morphological characters of different species of *Phoma*[33]

***Phoma betae* A.B. Frank Fig. 1[(1-4)]**

Synonyms: *Phyllosticta betae* Oudem, *Phoma sphaerosperma* Rostr., *Phyllosticta tabifica* Prill., *Phyllosticta spinaciae* H. Zimm., *Phoma spinaciae* Bubák & Willi Krieg., *Gloeosporium betae* Dearn. & E.T. Barthol.

Colonies with olivaceous brown felty aerial mycelium bearing irregular patches of grey to white, reverse greenish brown. Pycnidia globose to subglobose, 100–350) µ diam., densely covered by mycelial. Conidial matrix milky white, later rosy buff to ivory. Conidia straight or slightly curved, ellipsoidal, biguttulate or with two polar concentrations of many small guttules, aseptate, 6.9X3.5-4.5 µ.

Material examined: *P. betae*- ITCC 2238 (*Beta vulgaris* seeds, N.N. Khune, New Delhi, 1977) Fig. 1(5-6).

Synonyms: *Phoma ostiolata* V.H. Pawar, P.N. Mathur & Thirum.

Colonies with very little aerial mycelium, when present white and extremely thin. Pycnidia globose with 1–3 ostioles on a short neck, variable in dimensions. Conidial matrix grey to saffron. Conidia broadly or shortly ellipsoidal, often with a single guttule, 3.5–4.5X2.5–3µ.

Material examined: *P. capitulum*- ITCC 2086 (IARI, New Delhi, 1976), Fig. [1(7-10)]

Synonyms: *Sphaeria cucurbitacearum* Fr., *Laestadia cucurbitacearum* (Fr.: Fr.) Sacc., *Sphaerella cucurbitacearum* (Fr.: Fr.) Cooke, *Phyllosticta cucurbitacearum* Sacc., *Phyllosticta orbicularis* Ellis & Everh., *Ascochyta cucumis* Fautrey & Roum., *Phyllosticta citrullina* Chester, *Ascochyta bryoniae* Kabát & Bubák,

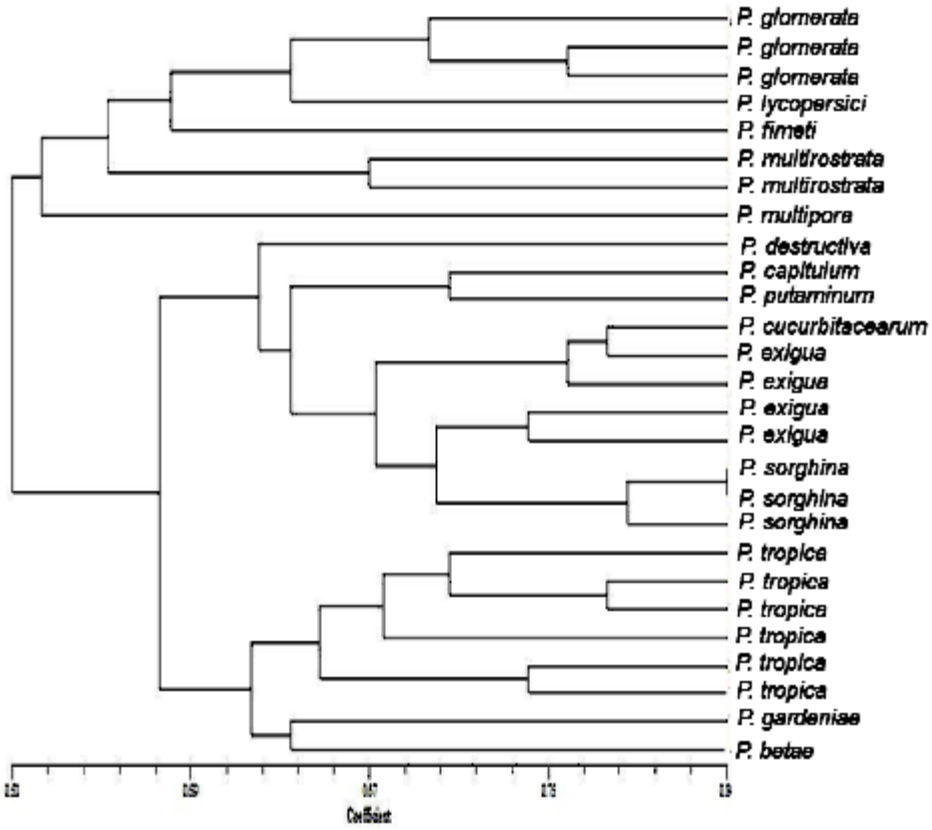

Fig. 2. Jaccard's similarity based dendrogram of *Phoma* isolates for RAPD markers

***Ascochyta melonis* Potebnia, *Ascochyta bryoniae* H. Zimm., *Diplodina cucurbitae* Nevod.**

Colonies regular, colourless to olivaceous grey; with woolly to floccose, white to olivaceous grey aerial mycelium; reverse buff green to olivaceous, to leaden grey or leaden black. Pycnidia globose to irregular, with 1(–2), sometimes papillate ostiole(s), later developing into an elongated neck, 80–380 µm diam., glabrous or with mycelial outgrowths. Conidial matrix white to buff. Conidia variable in shape, subglobose to ellipsoidal or allantoid, with several small guttules, usually aseptate, 4–8X 2–3 µm, but some larger 1-septate conidia may be present, up to 10 X4.5 µm.

Material examined: *P. cucurbitacearum* - ITCC 2087 (IARI, New Delhi,, 1976)

***Phoma destructiva* Plowr., Gard. Chron. Fig. 1** [11-14]

Synonyms: *Diplodina destructiva* (Plowr.) Petr., *Phyllosticta lycopersici* Peck

Colonies variable, usually with sparse aerial mycelium, but when present floccose and dark with patches of grey to whitish grey, often sectoring; reverse dark brown, often with a vinaceous tint. Pycnidia globose to irregular, solitary or confluent, glabrous with 1–3 papillate ostioles, honey/citrine to olivaceous, later olivaceous black. Conidial matrix buff to saffron coloured. Conidia 3.5-5X2 µm, consistently biguttulate, straight, cylindrical, ellipsoid or slightly irregular, reputedly produced in saffron tendrils.

Material examined: *P. destructiva* - ITCC 4305 (R.P. Gupta, Nasik, 1992); ITCC 6235 (K.E. Lawande, Raigurunagar, Pune, 2007)

***Phoma exigua* Desm. Fig. 1** [15-17]

Synonyms[14, 2-4, 33]

Colonies very variable with scalloped or lobed margin, usually with dense felty white, black or dark olivaceous aerial mycelium, not concentrically zoned. Pycnidia globose to subglobose or irregular with usually 1(–2) non-papillate ostiole(s), 75–200 µm diam., glabrous, solitary or confluent. Conidial matrix white to yellowish or rosy buff/salmon or rosy vinaceous. Conidia variable in shape and dimensions, subglobose, ellipsoidal to oblong, or allantoid, usually with two guttules, mainly aseptate, 5.5-10X2.5-3.5 µm, and becoming 1 septate.

Material examined: *P. exigua* - ITCC 2049 (IARI, New Delhi,, 1976); ITCC 2062 (T.P. Bhowmik, New Delhi, 1976); ITCC 2478 (V.Ravindra Nath, Hyderabad, 1977), ITCC 3601 (R.M. Saxena, Tannery effluent, Agra, 1986); ITCC 5546 (K. Bahadur, Large Cardamom, Kalimpong, 2004)

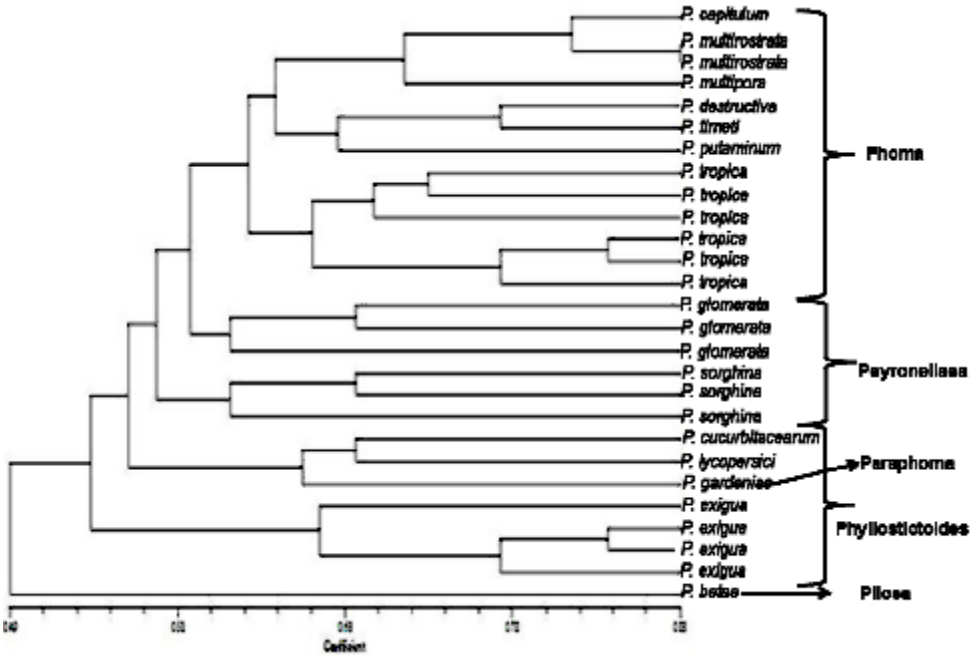

Fig. 3. Jaccard's similarity based dendrogram of *Phoma* isolates for ISSR markers

***Phoma fimeti* Brunaud Fig. 1** [18-21]

Colonies olivaceous brown or occasionally whiter, aerial mycelium moderately dense to dense, not concentrically zoned, reverse yellow to saffron on Oat meal. Pycnidia subglobose with 1 conspicuous somewhat papillated ostiole, 60–200 µm diam., glabrous, solitary or confluent. Conidial matrix whitish to ochraceous. Conidia3-3.5X2-2.5 µm, frequently with a large guttule, ellipsoid.

Material examined: *P. fimeti* - ITCC 2088 (IARI, New Delhi,)

***Phoma gardeniae* (S. Chandra & Tandon) Boerema Fig. 1** [22-24]

Synonyms

***Pyrenochaeta gardeniae* S. Chandra & Tandon**

Colonies regular, grey olivaceous to greenish olivaceous towards margin or colourless with grey olivaceous to dull green sectors, with finely floccose, grey olivaceous to olivaceous grey aerial mycelium. Pycnidia setose, globose to irregular with usually 1 slightly papillate or nonpapillate ostiole, 50–180 µm diam., olivaceous to olivaceous black; setae relatively short, up to 100 µm, concentrated around ostiole. Walls made up of 3–8 layers of cells, or filling nearly the entire cavity, outer layers pigmented. Conidial matrix white or flesh. Conidia ellipsoidal to ovoid, with or without small guttules, 5–8.5X 2–3.5 µm. Chlamydospores globose to subglobose, usually intercalary, solitary or aggregated, 6–15 µm diam., ochraceous to olivaceous with greenish guttules. Material examined: *P. gardeniae* - ITCC 4554 (*Gardenia resinifera*, Junagarh, V.A. Patel, 1995)

***Phoma glomerata* (Corda) Wollenw. & Hochapfel Fig. 1** [25-27]

Synonyms [18-22, 13, 33]

Colonies extremely variable, inasmuch as formation of pycnidia, aerial mycelium and chlamydospores and size and pigmentation of pycnidia, conidia and chlamydospores is influetced by the C/N ratio of the medium and the age of culture. Aerial mycelium sparse, although cultures often sectoring to produce olivaceous grey, grey white or brown strains of dense aerial mycelium. Pycnidia subglobose to obpyriform, 100–300 µm diam., papillate or with necks of various length, usually solitary but sometimes coalescing. Pycnidial wall three to five layers thick; the outer portion composed of more or less isodiametric but rounded and sometimes inflated cells with dark extracellular deposits. Conidial matrix at first rosy buff to salmon, later darkening and becoming olivaceous-brown. Conidia variable in shape and dimensions, mostly ovoid-ellipsoidal, sometimes slightly curved, 5-9X2.5-3 µm, hyaline but with age becoming pale olive-brown and minutely roughened. Chlamydospores brown, catenate, *Alternaria*-like, with transverse and longitudinal septa.

Material examined: *P. glomerata* – ITCC 3712 (Ficus twigs, S.P.Lal, New Delhi, 1987); ITCC 6236 (Ficus religiosa, S.W.Khadke, Waghapur, 2007); ITCC 6949 (Jamun tree, R.J. Jat, Jobner, 2011)

***Phoma lycopersici* Cooke Fig. 1** [28-31]

Synonyms: *Sphaeronaema lycopersici* Plowr., *Ascochyta lycopersici* Brunaud, *Ascochyta socia* Pass., *Diplodina lycopersici* Hollós, *Phoma ferrarisii* Cif., *Diplodina lycopersicicola* Bond.-Mont.

Colonies regular, colourless/olivaceous buff to grey olivaceous, with white to olivaceous grey/grey olivaceous aerial mycelium; reverse grey olivaceous/olivaceous grey to olivaceous, olivaceous buff near margin. Pycnidia globose to subglobose, with 1(–3) non-papillate or slightly papillate ostiole(s), 70–200 µm diam., glabrous or with short mycelial outgrowths. Conidial matrix whitish to buff. Conidia mainly aseptate, variable in shape, subglobose to ellipsoidal or allantoid, with several small guttules, 5–8.5X 2–3.5 µm, 1-septate conidia up to 15.5X4.5 µm.

Material examined: *P. lycopersici* – ITCC 6962 (Tomato leaves, M.N. Bhat, NCIPM, New Delhi, 2011)

***Phoma multipora* V.H. Pawar *et al.* Fig. 1** [32-33]

Colonies on regular with weakly undulating outline, with scarce, more or less felted white to olivaceous grey aerial mycelium, colony characterized by apricot to scarlet pigment; reverse similar. Pycnidia globose, usually with one or two wide ostioles (only incidentally 'multi'-ostiolate pycnidia occur), 120–300 µm in diam., glabrous, but the ostioles sometimes surrounded by short hyphae swollen at the tips ('setose' appearance), solitary or confluent in groups of 2–5 pycnidia. Conidial matrix rosy buff or vinaceous. Conidia broadly ellipsoidal, eguttulate or with a few small polar guttules, mostly 3.5–5X1.5–2.5 µm.

Material examined: *P. multipora* – ITCC 2090 (IARI, New Delhi, 1976)

***Phoma multirostrata* (P.N. Mathur *et al.*) Dorenb. & Boerema Fig. 1** [34-36]

Synonyms: *Sphaeronaema multirostratum* P.N. Mathur, S.K. Menon & Thirum. apud Mathur & Thirumalachar, *Phoma ushtrina* T.R.N. Rai & J.K. Misra

Colonies regular, colourless to weak olivaceous, with poorly developed, felted, white to grey olivaceous aerial mycelium or without any; reverse olivaceous. Pycnidia globose to subglobose or irregular, in this type variety (soil isolates) frequently with some variously shaped necks (up to 240 µm) and several ostioles, where not located at the tips of necks, papillate or non-papillate, always relatively large, usually more than 550 µm diam., glabrous, solitary and confluent. Conidial matrix whitish to cream or buff coloured. Conidia oblong to ellipsoidal, sometimes eguttulate but usually with 2–3 small or large polar guttules, variable in dimensions, mostly 5-6.5X2-2.5 µm. Chlamydospores (common in older cultures) oblong to ellipsoidal, in chains or clustered, often intercalary, but also terminal, olivaceous with green guttules, 5–15 µm diam.

Material examined: *P. multirostrata* – ITCC 2251 (Soil, N.N. Khune, Jodhpur, 1977), ITCC 6031 (Catharanthu roseus, V.L. Krishna Murthy, Shimoga, 2005), Fig. 1 [37-39]

Synonyms: *Aposphaeria putaminum* (Speg.) Sacc., *Coniothyrium putaminum* (Speg.) Kuntze, *Phoma radicicola* McAlpine, *Phoma dunorum* ten Houten

Colonies comparatively fast growing, with little aerial mycelium, although when present olivaceous to grey; reverse buff to apricot. Pycnidia globose, mostly with a short neck, 60–300 µm diam., covered with hyphal strands (hairy), mostly solitary but also confluent. Conidial matrix whitish/ salmon. Conidia broadly ellipsoidal, mostly single guttule, larger ones often biguttulate, 2.5-4.5X2-2.5 µm.

Material examined: *P. multirostrata* – ITCC 2164 (Soil, C.P. Agarwal, Assam, 1970).

***Phoma sorghina* (Sacc.) Boerema *et al.* Fig. 1** [40-44]

Synonyms [19-22,33]

Colonies extremely variable, usually with fluffy to dense aerial mycelium wich is basically grey to green to olivaceous or darker, but with very characteristic white to salmon pink tinges or area; reverse often reddish. Pycnidia subglobose, 50–200 µm diam., usually with a distinct straight or somewhat curved neck up to 80 µm long, occasionally touching but usually not confluent. Conidial matrix usually salmony in colour. Conidia 4-5X2-2.5 µm, ellipsoid, eguttulate. Chlamydospores highly variable and irregular, uni- or multicellular, or *Alternaria*-like, mostly intercalary, sometimes terminal-lateral, solitary or in chainswith 8–35 µm diam.

Material examined: *P. sorghina* – ITCC 2428 (Ravindra Nath, Hyderabad, 1977), ITCC 3176 (Brinjal, Bhawani Prasad, Simla, 1982), ITCC 4160 (Paddy, F.Khan, Bhopal, 1991), Fig. 1 [45-47]

Synonyms: *Sphaeronaema coloratum* P.N. Mathur & Thirum.

Colonies with floccose aerial mycelium at first, later becoming wooly, pale olivaceous grey to chocolate brown, with an olivaceous to black reverse. Pycnidia subglobose, with 1–5 conspicuous dark circumvalated ostioles, 100-400 µm diam., glabrous, solitary. Conidial matrix white yellowish. Conidia ellipsoidal, with 2(3) distinct guttules, 3-4X1.5 µm.

Material examined: *P. tropica* – ITCC 2258 (N.N. Khune, New Delhi, 1976), ITCC 2811 (Brinjal phyllosphere, V.K. Tyagi, Ujjain, 1976), ITCC 2812 (Brinjal phyllosphere, V.K. Tyagi, Ujjain, 1976), ITCC 3765 (Kulthi, Bisht, New Delhi, 1987), ITCC 3767 (Soybean, Bisht, New Delhi, 1987), ITCC 3796 (*Momordica charantia*, S.M. Reddy, Warangal, 1988), ITCC 6206 (*Dolichous lablab* stem, N.A.U. Navsari, 2007)

Genetic diversity analysis of different isolates of *Phoma* species

RAPD

The isolates of *Phoma* species were subjected to RAPD analysis where 6 random primers were used for *Phoma* species resulted in robust and reproducible RAPD fragment patterns. The selected primers generated 499 RAPD bands against 6 random primers used in *Phoma* species, out of which OPA-3 primer was produced maximum number of bands (98 bands) followed by OPA-2 (93 bands), OPO-4(81 bands), OPE-14 (79 bands), OPA-11 (76 bands) and OPN-20 (72 bands) and the size of the amplification products ranged from 250 bp to 10 Kb. The percent of polymorphism ranged from 80 to 100%.

The genetic similarity between the isolates of *Phoma* species was determined on the

basis of Jaccard's similarity coefficient. The mean value of the Jaccard's similarity coefficient of the RAPD marker for *Phoma* species was 0.67.The highest genetic similarity was observed between the different isolates of *P. sorghina* showing 84% and the least was between the isolates *P. multipora* are showing 53%.

The dendrogram generated using unweighted pair group method arithmetic (UPGMA) based on NTSYS pc version 2.02i software resulted in three major clusters with similarities ranging from 53 to 84%. Cluster I: Consists of 8 isolates, *P. fimeti* (2088), *P. glomerata* (3712, 6236, 6949), *P. lycopersici* (6962), *P. multirostrata* (2251, 6031) and *P. multipora* (2090), these isolates are sharing genetic similarity ranging from 0.53 to 0.75. Cluster II: consists of 11 isolates, *P. cucurbitacearum* (2087), *P. exigua* (2049, 2478, 3601, 5546), *P. capitulum* (2086), *P. destructiva* (6235), *P. putaminum* (2164), *P. sorghina* (2428, 3176, 4160), these isolates are sharing genetic similarity ranging from 0.58 to 0.84. Cluster III: contains 8 isolates, *P. gardeniae* (4554), *P. tropica* (2258, 2811, 3765, 3767, 3796, 6206) and, *P. betae* (2238). These isolates are sharing genetic similarity ranging from 0.62 to 0.80 (Fig.1). This dendrogram revealed the presence of the variability among the different isolates of *Phoma* spp. by separating them in different cluster.

ISSR Markers

Five ISSR primers were screened and only four of them gave satisfactory amplification and band resolution from 25 isolates were taken for the study. The selected primers generated 571 ISSR bands and the size of the amplification products ranged from 250 to 1 kb. Out of 4 selected primers, ISSR-1 primer was produced maximum number of bands (162 bands) followed by ISSR-3 (152 bands), ISSR-2 (132 bands) and ISSR-4 (125 bands) respectively.The percent of polymorphism ranged from 80 to 100% .

The mean value of the Jaccard's similarity coefficient of the ISSR marker was 0.68. The dendrogram based on the ISSR sequence data separated the isolates of *Phoma* species into 5 major clusters with similarities ranging from 49 to 88% (Fig. 3). Cluster I: consist of 13 isolate *P. capitulum* (2086), *P. destructiva* (6235), *P. fimeti* (2088), *P. multipora* (2090), *P. multirostrata* (2251, 6031), *P. putaminum* (2164), *P. tropica* (2258, 2811, 3765, 3796, 3767, 6206), these isolates are sharing genetic similarity ranging from 0.64 to 0.88..Cluter II: consist of three isolates, *P. glomerata* (3712, 6236, 6949), these isolates are sharing genetic similarity ranging from 0.62 to 0.69. Cluster III: consist of three isolate, *P. sorghina* (2428, 3176, 4160), these isolates are sharing genetic similarity ranging from 0.54 to 0.72. Cluster IV: consist of three isolates, *P. gardeniae* (4554), *P. cucurbitacearum* (2087), *P. lycopersici* (6962), these isolates are sharing genetic similarity ranging from 0.52 to 0.69. Cluster V: consist of four isolates *P. exigua* (2049, 2478, 3601, 5546), these isolates are sharing genetic similarity ranging from 0.50 to 0.84. Cluster VI: consisting only one isolate *P. betae* (2238), was stood into separate cluster sharing genetic similarity was 0.49 (Fig.2).

ISSR primers were able to separate the *Phoma* species isolates on the basis their section *viz*. Paraphoma, Peyronellaea, Phoma, Phyllostictoides and Pilosa. Only one species, *P. gardeniae* was not cluster with other isolates of same section. The dendrogram obtained separated *Phoma* speciea isolates into 5 groups having large amount of variability. ISSR markers were found a much more efficient tool to differentiate variability among the different isolates of *Phoma* species.

DISCUSSION

The purpose of study was to study the forteen different agriculturally important *Phoma* species based on typical morphological charcterstics for developing identification key(s) and genetic relationship among different isolates of *Phoma* spp. Morphology of different *Phoma* species were studied based on growth and pigmentation of cultures, shape and size of pycnidia, condia, chlamydospores and sclerotia. Each species of *Phoma* isolates were clearly discussed in the area of taxonomy description. *Phoma* and *Phoma*-like fungi were thoroughly studied and calssified by Boerema and his colleagues[1, 4-34, 37-41, 46]. Few *Phoma* species of Indian origin *viz., P. pinodella, P. pomorum, P. herbarum, P. exigua, P. tropica, P. sorghina, P. capitulum, P. jolyana, P. fimeti, P. chrysanthemicola, P. complanata, P. destructive, P. eupyrena* and *P. arachidicola*, were defined based on cultural and morphological studies[48-65]. Therefore present study

will supliment with all the above studied by providing a authentic indentification key for identification of agriculturallu important *Phoma* species.

The twenty-seven geographically diverse isolates (forteen species) of the morphological species *Phoma* were invariant in their RAPD and ISSR studies. RAPD has been used widely as an effective molecular tool to evaluate genetic variance at or below the species level. In our RAPD analyses different species and isolates of the genus *Phoma* produced different DNA profiles on the gels denoting significant intraspecific genetic variation. Based on RAPD results 51 -84 % of genetic variations showed among forteen different *Phoma* species. All the twenty seven isolates belongs to five sections, different sources and places. The RAPD results showed the polymorphism within the particular *Phoma* species. Zhou *et al*., (2005)[69] also studied the genetic varation of *P.lingam* isolates through RAPD analysis. Among these three isolates of *P. lingam* significantly different based on the conbfidence intervals used for bootstrap analyses. While RAPD markers cover the whole genome for amplification, ISSR amplifies the region between 2 microsatellites. Hence, the polymorphisms reflect the diversity of these regions of the genome[45]. ISSR studies clearly separated 25 isolates of *Phoma* spp. into five different sections. *P. gardenia* belongs to paraphoma section associated with the Phyllostictioides section. ISSR showd 49-88% genetic varation, where as RAPD showed only 51-84% varation without any proper classification among the forteen species of twenty seven different isolates. According to Gruyter *et al*.[36] the classification of species in *Phoma* and allied genera is still controversial. Confusing characters are reported among several Phoma sections and related genera such as Ascochyta, Asteromella, Microsphaeropsis, Phomopsis, Phyllosticta, Pleurophoma, Pyrenochaeta and Stagonospora. But in our ISSR results clearly separted four different sections of forteen different *Phoma* species based on their sections.

In overall we described typical characters and key for the identification of of forteen different *Phoma* species based on morphology. ISSR revealed higer level of genetic vartion comparatively RAPD among the isolates. ISSR clearly separted five different section of *Phoma* species. The genetic diversity of these isolates studied by ISSR markers is prerequisite for developing a diagnostic tool for the identification and differentiation of *Phoma* and other allied genera.

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