Molecular Identification and Variability of *Pestalotiopsis microspora* of Coconut by RAPD marker

A THESIS

BY

FARHANA AFROZE PREYA

STUDENT ID. MS-190803

A Thesis (Course No. PLP-6104) Submitted to Agrotechnology Discipline in Partial Fulfillment of the Requirements for the Degree of

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IN

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AGROTECHNOLOGY DISCIPLINE

LIFE SCIENCE SCHOOL

KHULNA UNIVERSITY

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Abstract

Coconut (*Cocos nucifera L.*) is one of the most important fruit trees in Bangladesh. This tree is susceptible to various pathogens. This study was aimed for molecular identification and variability of *Pestalotiopsis microspora* of leaf spot disease of coconut. An experiment was conducted with thirteen *Pestalotiopsis microspora* isolates during the period from January 2021 to January 2022 in the Plant Protection Laboratory of Agrotechnology Discipline at Khulna University, Khulna. Isolates were isolated from infected coconut leaves collected from different locations in Khulna division. Among 13 isolates, three isolates (Pm2, Pm8 and Pm11) were randomly selected for molecular identification. ITS region of the pathogenic fungi was sequenced and the tested isolates were identified as *Pestalotiopsis microspora*. In this study, isolated fungi were showed 100% similarity with *Pestalotiopsis microspora* following GenBank references (OK254035.1, OK254041.1 and AY681483.1). In molecular variation total seven RAPD primers were used and generated clearly visible banding patterns. Among the 13 isolates, a maximum of 88% dissimilarity was found between Pm9 and Pm13 isolates. Pm9 and Pm13 isolates were collected from Koia bazar, Harintana and Batiaghata, Dumuria under Khulna division. Cluster analysis showed two major clusters. Cluster I and II was further sub-divided into two sub cluster. From this PCA analysis it also disclosed that positive co-relation was among the Pm1, Pm2, Pm3, Pm5, Pm6,Pm11, Pm12 and Pm13 isolates and negative co-relation was belonging among the Pm4, Pm5, Pm8, Pm9 and Pm10 isolates.

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June 2022 **The Author**

DEDICATED TO MY BELOVED PARENTS

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Chapter I

INTRODUCTION

Coconut (*Cocos nucifera L.*) is a perennial fruit crop that belongs to the family Arecaceae. Coconut palms are successfully grown in the tropical countries of the world and are hence referred to as "King of the tropical palms" (Hajieghrari et al., 2008). In Bangladesh, it is mostly grown in the southern part. Its production in Bangladesh was 92,417 MT from 43,000 ha of land during 2017–2018 (BBS, 2018).

Coconut is susceptible to various foliar diseases. Among these, leaf spot (*Pestalotiopsis microspora*) is one of the most important diseases in coconut (Elliott et al., 2004). The palms become more susceptible to *Pestalotiopsis* at the early stage (5–10 years old). The disease advanced with the date of maturity of the palms (Karim, 2005). In Bangladesh, leaf spot appears almost in every coconut producing area and plays a vital role in a low level of coconut production. In the coconut growing areas of Bangladesh, the symptoms of leaf spot were yellowish brown spots with gray brown margins on the mature leaves (Hajieghrari et al., 2008). *Pestalotiopsis microspora* is the causative agent of a fungal disease of coconut. The fungus causes leaf spots, petiole/rachis blights and sometimes bud rot of palms. *Pestalotiopsis microspora* attacks all parts of the leaf from the base to the tip whereas most diseases only infect the leaf blade or the leaf petiole (Elliott and Monica, 2015).

The ability of the genus of *Pestalotiopsis* to infect different hosts in different temperature. Temperature strongly influences the mycelial growth, synthesis of metabolites, and morphological characters of *Pestalotiopsis spp.* (Vegh and LeBerre, 1992; Zhiquan et al., 1999). *Pestalotiopsis* contains five celled conidia (including three median cells) with a single hyaline basal appendage and two to four hyaline apical appendage. The three median cells are thick walled and light to dark brown, whereas the apical and basal cells are hyaline

(Maharachchikumbura et al., 2014). Jeewon et al. (2003) identify the fungal isolates based on molecular characterization and phylogenetic analysis.

Molecular identification has been proposed as a complementary tool to the morphological identification of microbial strains. *Pestalotiopsis spp.* isolates were identified by sequencing the internal transcribed spacer (ITS). Identification methods were compared to indicate the key morphological characters for species characterization (Bonito et al., 2011). A molecular genetic marker is a gene or DNA sequence used to distinguish various species variants. Markers based on DNA are classified as non-PCR-based and PCR-based markers. PCR based sequencing technique is the simplest, fastest and appropriate ways of molecular identification (Elliott et al., 2004).

Large number of molecular markers has enabled precise characterization of fungal strains. RAPD is very important because of its larger stability, potency and accuracy compared to morphology (Williams et al., 1990). In many cases, RAPD techniques have been used for the identification of individuals in different populations and distinguishing isolates of various fungi (White et al., 1990). As these techniques are simple, inexpensive and do not require any sequence information, they are widely followed in molecular studies. Non-PCR-based markers are informative but practical involvement of radioactive isotopes, high price, absolute sequence data requirement and advanced laboratory limits on their use (Mullis 1994, Saiki et al., 1988). RAPD is a method based on polymerase chain (PCR) in which random DNA fragments are amplified with single primers of random nucleotides with sequence (Williams et al., 1990; Hilton et al., 1997). RAPD chain polymerase reaction is considered helpful in obtaining genetic data (Singh et al., 2009). RAPD-PCR is an effective tool used in genetic research for microorganism's molecular genetic diversity. The RAPD markers are genetically reliable and have been commonly used in plant's (Grimmer et al., 2007). The RAPD technique is easy, sensitive and quick to detect, distinguish, and determine

the phylogenetic relationship between isolates of multiple pathogen species (Narayanasamy, 2001). However, in the band patterns RAPD-PCR has a tendency for a reproducibility issue. It is possible to use RAPD-PCR to study fungal taxonomy.

Considering the above facts the objective of the study were

- To identify the *Pestalotiopsis microspora* isolates obtained from infected coconut leaves by sequencing of ITS region
- To determine the genetic variability among the isolates of *Pestalotiopsis microspora* by RAPD analysis

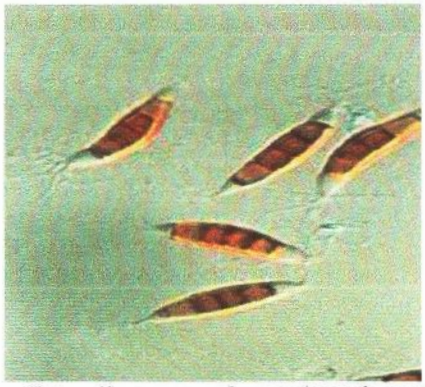
Chapter II

Review of Literature

2.1 The pathogen

Pestalotiopsis microspora was the cause of the most common leaf spot of coconuts and noted distinct differences between lesions associated with *P. microspora* and with three other Pestalotiopsis species on coconuts (Menon & Pandalai, 1960). *Pestalotoiopsis* Species is an appendage-bearing conidial anamorphic form (coelomycetes) in the family Amphi-sphaeriaceae (Barr, 1975). Species of *Pestalotiopsis* are common in tropical and temperat ecosystems (Bate-Smith & Metcalfe 1957) and may cause plant disease are often isolated as endophytes or occur as saprobes (Liu et al., 2008a). This species is characterized by 6-celled conidia with four deeply olivaceous central cells, distosepta, hyaline terminal cells and simple or branched appendages arising from the apex (Fig. 1). Steyaert (1949) revised *Pestalotia* and divided the genus into three main groups based on the conidial forms. *Pestalotiopsis* was considered to be a monophyletic genus and Steyaert (1949) suggested that the type species could be distinguished from *Pestalotiopsis* by it cupulate conidiomata and distoseptate median cells. Steyaert (1949) further divided Pestalotiopsis into additional sections based on the number of apical appendages. These were the Monosetulatae, Bistulatae, Trisetulatae and Multisetulatae, which were further divided into subdivisions. Conidia with a single setulae (apical appendage) were included in the monosetulatae, which was further divided into forms with simple and branched setulae. Conidia with two setulae or on average two setulae were included in the bistulatae. Conidia with three setulae or onaverage three setulae were included in the trisetulatae which was further divided by concoloros or versicolors conidia, fusiform or claviform and spatulate or non-spatulate setulae. Conidia with more than three setulae were included in the multisetulatae. (Steyaert, 1949). Jeewon et al., (2003) and Tejesvi et al.,

(2009) compared morphology with sequence data and showed that species of *Pestalotiopsis* display considerable diversity in morphology and that isolates grouped together based on similarities in conidial morphology.



Source: (https://www.google.com/search-conidia-pestalotia)

Figure 1. ***Pestalotiopsis microspora*** conidia

2.2 Life/disease cycle

A disease cycle of a pathogen may be closely related to its life cycle, and the former refers to the emergence, development and maintenance of the disease (Agrios, 2005). Species of *Pestalotiopsis* are not highly host-specific and taxa may have the ability to infect a range of hosts (Hopkins & McQuilken 2000 and Keith et al., 2006). Pathogenic species of *Pestalotiopsis* initially make contact with the host where the infection occurs (inoculum), probably by means of the conidia or fragmented spores (Espinoza et al., 2008). These inocula may survive during harsh weather conditions and may cause primary infections. Secondary inoculum produced on diseased tissue may cause secondary infections and increase the severity of the disease. Watanabe et al., (2010) reported that Species of *Pestalotiopsis* have constantly been isolated as endophytes from plant tissues. The pathogenic phase may be triggered by a combination of environmental factors, plant susceptibility and the virulence of the pathogen. Madar et al., (1991) pointed that *Pestalotiopsis* is also considered as a weak pathogen and most weak pathogens penetrate the host through natural openings such as stoma, lenticels and hydathodes (Agrios, 2005). Wright et al., (1998) stated that species of

Pestalotiopsis only infect wounded or stressed plants, so pruning wounds or other physical means play important roles in disease development. High temperature, high rainfall and human activities may also trigger infections and this may lead to disease development (Hopkins & McQuilken 2000). The anamorph teleomorph relationships and life cycles are not well known for most species, as the sexual stage does not often develop (Armstrong-Cho & Banniza, 2006). Watanabe et al., (2000) studied conidial adhesion and germination of spores and showed that infection occurs in four stages. At the beginning, the lower median cell germinates. Future successive infections can be achieved by two upper median cells. In the first stage, weak adhesion is achieved by the mucilaginous matrix coating the conidia. The next two stages provide a strong attachment by release of fibrillar adhesive substances. In the third stage, fibrillar adhesive substances are produced along the length of the pedicel to the apex of the basal cell and at times a smaller amount of fibrillar material is released from the apical appendages. The fourth stage involves the release of fibrillar material at the point of germ tube emergence. NagRag, (1993) described conidiomata of the genus as variable ranging from acervuli to pycnidia. A general disease cycle for *Pestalotiopsis* is isillustrated in Fig. 2.

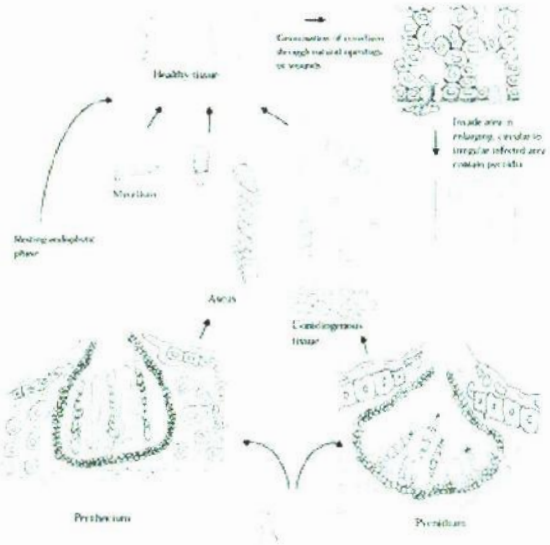


Figure 2. Disease cycle of the genus *Pestalotiopsis* (Von Arx, 1974)

2.3 Host range of *Pestalotiopsis microspora*

The fungus has been isolated from a wide variety of palm tissue. The fungus is not host specific so the disease could be present in many more types of palm. The fungus has also been known to infect date palm (*Phoenix roebelenii*) and has been a big problem in Florida during the winter months (Keith et al., 2006). *Pestalotiopsis spp.* not only infects the coconut but also infect several other palm trees such as betel nut, palmyra palm, date palm, oil palm and ornamental palms (Karim, 2005). Elliott et al., (2004) stated that Some varieties of bananas have also shown symptoms of the disease, but it is unclear if it is in fact *Pestalotiopsis microspora*. Agrios (2005) as indicated previously, *Pestalotiopsis* can be readily isolated from healthy palm tissue. It has been demonstrated that the fungus usually requires wounds for the plant penetration (infection) necessary for disease development.

2.4 Dispersal

Watanabe et al. (2000) studied the spore of *Pestalotiopsis* is considered to be a dry spore. Elliott et al. (2004) stated that *Pestalotiopsis* may produce large numbers of spores which are easily dispersed in air or by water splash. These spores are spread by rain, overhead irrigation, wind, pest activity, and human activity. Water is not only critical for spread of the spores, but it is also critical for spore germination and infection (invasion) of the leaf tissue (Keith et al., 2006).

2.5 Survival and occurrence of *Pestalotiopsis spp.*

Pestalotiopsis spp was able to survive on infected plant debris for up to 1 year to initiate new infections (Nag Raj, 1985). The source of the inoculum can be wild plantations (Keith et al., 2006), flowers, crop debris, disease stock plants, used growing media, soil and contaminated

nursery tools (McQuilken & Hopkins, 2004), splashed water droplets (Hopkins & McQuilken1997; Elliott et al. 2004) and also spores in the air (Xu et al., 1999). Aly et al., (2010) reported that species of *pestalotiopsis* surviving as endophytes in plant tissue. Several *Pestalotiopsis* species produce thermo tolerant conidia which survive exposure to dry heating (temperature above100°C) for several hours (Suryanarayanan et al., 2011a).

2.6 Worldwide distribution of *Pestalotiopsis spp.*

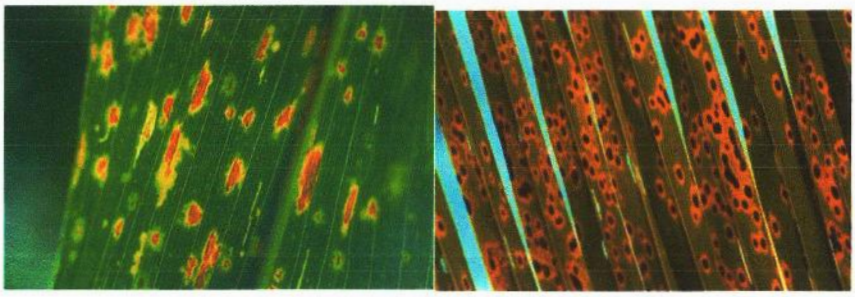
Pestalotiopsis like fungi cause diseases on many different species of plants worldwide (Chamorro et al., 2016). Elliott et al. (2004) reported that *Pestalotiopsis* species are broadly distributed in the world, occurring on a wide range of substrata. Its species have a worldwide distribution, particularly in tropical and temperate ecosystems. Most species are plant pathogens (Zhu et al., 1991) and some are saprobes in soil (Agarwal and Chauhan, 1988) and in plant debris (Osono & Takeda, 1999). *Pestalotiopsis microspora* was reported for the first time from British Guyana and later from Malaysia, Sri Lanka, New Hebrides, India, and Trinidad. Studies on *Pestalotiopsis* diversity in China has revealed about 120 pathogenic and saprobic species with some new species and new combinations (Zhu et al., 1991 and Chen et al., 2002).

2.7 Symptoms of Leaf Spot disease

In general, the initial symptom for leaf spots is a small, oval up to 1.5 cm long, grey with a dark brown border and water-soaked lesion. Within days, this lesion turns to varying shades of yellow, gray, reddish-brown, brown, or black. If the disease is restricted, the spots may never be more than 1/4 inch in size (Elliott et al., 2004). The colored lesions may be surrounded by a halo or ring of tissue that is a different color. For example black spots surrounded by chlorotic (yellow) halos; yellow spots surrounded by reddish-brown rings or black rings; gray spots surrounded by a black ring, which is surrounded by a chlorotic halo.

The common theme is that there is a disruption in the normal, uniform color of the leaf blade and that disruption is round or oblong in shape (Figure 3) (Chase, 1993).

If environmental conditions are optimum for the disease, the spots can increase in number or size such that large portions of a fan palm blade or large number of leaflets of a feather palm blade are diseased (Broschat & Elliott, 2005). If severe enough, entire leaflets or leaves may desiccate and die as lesions expand and coalesce, causing leaf blights. Leaf spots usually appear on leaves of all ages. With most leaf spot diseases, the pathogen will produce spores directly on the leaf tissue.



Source: (https://www.google.com/search?q=coconut+grey+leaf+spot)

Figure 3. Leaf spots often change in color and size as the disease progresses

2.8 Epidemiology of leaf spot

Conidia produced in infected plant debris from the previous infected crop provide the primary inoculum to infect the plant (Beckman & Payne, 1982; Latterell & Rossi, 1983 and Payne & Waldron, 1983). Spore counts were highest in early afternoon but aerial concentrations of conidia remain low until sporulating lesions on plants are formed. These low conidial counts, however, are not the reason for initial slow development of disease. It is rather the absence of suitable environmental conditions that delay disease development in the early season (Payne & Waldron, 1983). High relative humidity (in excess of 90 to 95%), and temperatures of 22 to 30°C are considered optimum for spore germination (Beckman & Payne, 1982 and Rupe et al., 1982). In laboratory studies, Rupe et al. (1982) found that spores

require at least 6 hours of continuous leaf wetness for germination and optimum conditions were 9 hours of leaf wetness, at temperatures between 18 and 25°C. The conidia were unable to survive wetting and drying during germination and must germinate in an uninterrupted period of wetness and also found germination in vitro to occur after 6 hours with free water. Beckman and Payne (1982), on the other hand, were able to germinate conidia on plants after 24 hours at 22 to 30°C. Tillage systems that leave sufficient previously diseased crop residue on the soil surface provide sufficient primary inoculum to produce severe levels of gray leaf spot (de Nazareno et al., 1993).

2.9 Yield loss of coconut due to leaf spot disease

Leaf spot must damage leaves at or just after growth stage to cause severe yield reduction. Blighting of the leaves above the ear leaf leads to severe yield losses exceeding 50%. (Elliott et al., 2004). Steyaert, (1949) pointed that Leaf spot does not occur until well into the grain fill period probably results in very little yield loss. Research has shown that there is not a direct relationship between the level of disease and yield loss. Several factors may contribute to this response, including yield potential of the hybrid, and the ability of leaf reduce chlorosis. de Nazareno et al. (1993) reported that the disease is capable of reducing yields by as much as 60% in the more humid, high potential growing areas.

2.10 Morphological characteristics of *Pestalotiopsis spp*

Morphological characters used to differentiate species of *Pestalotiopsis* and similar genera are limited (Hu et al., 2007). Egger (1995) described that the morphological characters are plastid and morphological markers vary between host and environment. Hu et al. (2007) showed that conidial morphology is highly variable within isolates of *Pestalotiopsis*. Conidial morphology is the most widely used taxonomic character for the genus *Pestalotiopsis*. Most species are divided into different groups based on the size of the conidia. The length and

width are good taxonomic markers for the genus and stable within the different media and the generations in most cases (Hu et al., 2007). Colour of the median cells is still a widely used character, and all species separate into three groups based on this- concolorous, versicolorous umber olivaceous and versicolorous fuliginous olivaceous. Molecular evidence indicates that it is more precise to group species according to concolorous and versicolorous rather than the above three groups (Jeewon et al., 2003). The length of the apical appendages and the number of the apical appendages are also widely used characters for species identification described by Barr (1975). Petrak (1947) reported that some species can also be identified by the presence of knobbed apical appendages. The apical appendages can arise from the top middle, bottom or different positions in the apical hyaline cells and such characters are widely used in species identification. Jeewon et al. (2003) and Tejesvi et al. (2009) compared morphology with sequence data and showed that species of pestalotiopsis display considerable diversity in morphology and grouped together based on similarities in conidial morphology. Hu et al. (2007) found that conidial characters such as conidial length, median cell length, conidial width and colour of median cells were stable characters within *Pestalotiopsis*; however, the length of the apical and basal appendages varied. Jeewon et al. (2003) evaluated the morphological characters that could be used to differentiate species of *Pestalotiopsis*.

2.11 Molecular characterization and identification

Kang et al. (1998) reported molecular studies have shown that Pestalotiopsis is monophyletic (Jeewon et al., 2002, 2003, 2004). Molecular studies have shown that the genus contain two distinct lineages again based on pigmentation of median cells and four distinct grouping based on morphology of apical appendages but the reliability of other phenotypic characters of this genus was not supported (Jeewon et al., 2003). Zhu et al. (2008) reported that diversity and identification of fungi had a great impact on fungal taxonomy due to rapid

developments in molecular techniques (Phillips et al., 2007 and Thongkantha et al., 2009). Fungal identification is more reliable when molecular approaches are combined (Hyde and Soytong, 2008 and Than et al., 2008). Sequence analysis of the Internal Transcribed Spacer (ITS) region of nuclear ribosomal DNA (rDNA) has been widely used for molecular identification and phylogenetic diversity studies of fungi (Egger, 1995; Lacap et al., 2003 and Promputtha et al., 2005). The ITS polymorphism might occur at genus, species or individual level making it useful for molecular studies (Carvalho et al., 2009). Vogler and Bruns (1998) pointed that the DNA sequences of ITS regions of rDNA gene envolves relatively quickly and can be useful in determining inter-species variations.

2.12 Variability in the population of *Pestalotiopsis spp.*

Most resent *Pestalotiopsis* research is based on endophytic isolates (Liu et al., 2006; Wei et al., 2007 and Watanabe et al., 2010) and has resulted in a four new species being described. Most genetic variablity was found between *P. macrochaeta*, *P. uvicola* and *P. pallidotheae*. Hu et al. (2007) reported that Variability was found on morphological characters and either gene sequence data. The diversity on the endophytic species of Pestalotiopsis is ubiquitous and is not largely influenced by geographical factors (Wei et al., 2007 and Tejesvi et al., 2009). Tejesvi et al. (2005) stated that the endophytic species of *Pestalotiopsis* dominant in the winter season and their colonization are comparatively low in the monsoon season. The colonization frequency of species of *Pestalotiopsis* increased with the increasing the age of the host plantand colonization frequency was variable (Wei et al., 2007).

2.13 Randomly amplified polymorphic DNA

Randomly amplified polymorphic DNA (RAPD) is a PCR-based technique which uses arbitrary primers which bind to the nonspecific sites on the DNA and amplify the DNA. These amplified fragments are then migrated on agarose gel and difference in the band

pattern is observed. This is relatively simple and less-expensive technique used for the diversity study (Williams et al., 1990). RAPD technology provides a quick and efficient screen for DNA sequence based polymorphism at a very large number of loci. The major advantage of RAPD includes that, it does not require pre-sequencing of DNA. The vast range of potential primers that can be used, give the technique great diagnostic power. The advern of Randomly Amptifred Potymorphic DNA (RAPD) by Williams et al. (1990) provided new tool for the molecular geneticist. Randomly amplified polymorphic DNA, or RAPD, marker analysis utilizes short PCR primers consisting of random sequences usually in the size range of 8 to 15 nucleotides in length. Complex patterns of PCR products are generated as these random sequence primers anneal to various regions in an organism's genome.

2.14 DNA barcoding marker for species level identification of fungi

With the availability of molecular data and advanced possibilities for phylogenetic analyses, Jeewon et al. (2003b) showed, on the basis of the generally accepted barcoding marker for fungi, the internal transcribed spacer (ITS), that *Pestalotiopsis* comprises three deep divergent lineages. This finding was integratively confirmed by Maharachchikumbura et al. (2011, 2012, 2013a) by comparison of morphological data and a multigene analysis including ribosomal and DNA barcoding marker.

2.15 Sexual and asexual stage of *Pestalotiopsis microspora*

Shearer et al. (2007) described that one fifth of all known anamorphic fungi lack known sexual states. *Pestalotiopsis* is a species rich in anamorphic genus with species mostly lacking sexual morphogenesis. The links between sexual and asexual stage are mostly from indirect evidence, with some links known through experimental or molecular data (Kendrick 1979; Reynolds, 1993). The asexual *Pestalotiopsis* state has been recorded in the *Pestalotiopsis microspora* (Barr 1975 and Nag Raj 1985). In the laboratory species of *Pestalotiopsis* rarely

develop sexual forms (Metz et al., 2000). Metz et al. (2000) obtained the sexual state of *P. microspora* on potato dextrose agar (PDA). However, this took 5 to 6 months of incubation. The asexual stage formed after 3–6 weeks on water agar with dried surface when incubated at 16–20 °C with 12 h of light per day and was identified as *Pestalotiopsis microspora.*

Chapter III

Materials and Methods

3.1 Experimental site and laboratory

The Experiment was performed in Plant Protection Laboratory and Horticulture Laboratory of Agrotechnology Discipline, Khulna University, Khulna, Bangladesh; from January 2021 to January 2022. Infected coconut leaf sample was collected from different locations of Khulna division.

Source: (https://en.shampratikdeshkal.com/zilla-news/news/210720652/covid-khulna-division)

Figure 4. Location of Sample collection area

3.2 Sample collection

Coconut leaf sample with distinct leaf spot symptom (Nanayakkara et al., 2008) was collected from different locations of Khulna division.

Table 1. Sample collection location of the study

SL. No.	Sample designation	Location/origin of the isolates
Isolate 1	Pm1	Pouroshovar mor, Thana- khalishpur, Division- khulna
Isolate 2	Pm2	Rupsha stand road, Thana- Rupsha, division- khulna
Isolate 3	Pm3	Itola, Upailla- Dumuria, Division- khulna
Isolate 4	Pm4	Keshari, Upazilla- Dumuria, Division- khulna
Isolate 5	Pm5	Chuknagar bazar, Upazilla- Chuknagar, Division- khulna
Isolate 6	Pm6	Dacop, Upazilla- Dumuria, Division- khulna
Isolate 7	Pm7	Rampal, Upazilla- Bagerhat sadar, Division- khulna
Isolate 8	Pm8	Narikel tola, Thana-Shatkhira sadar, Division- khulna
Isolate 9	Pm9	Koia bazar, Thana- Harintana, Division- khulna
Isolate 10	Pm10	Konda para, Thana-Bagerhat, Division- khulna
Isolate 11	Pm11	Noapara, Upazilla-Jashore, Division- khulna
Isolate 12	Pm12	Hazrapur, Upazilla-Magura sadar, Division- khulna
Isolate 13	Pm13	Batiaghata, Upazilla- Dumuria, Division- khulna

3.3 Preparation of Potato Dextrose Agar (PDA)

PDA was prepared following the standard procedure (Anonymous, 1968). 200 g peeled potato was cut into slice and boiled in 1000 ml distilled water. After boiling it was sieved into a beaker and 20 g dextrose was mixed with it, then 15 g agar was added with it. The beaker was then placed on a heater with magnetic stirrer and then adjusted to 1000 ml by distilled water. After melting the prepared medium was sterilized in an autoclave at 121°C temperature for 20 minutes.

Figure 5. Prepared PDA

3.4 Isolation of *Pestalotiopsis microspora*

The fungus was Isolated according to the method of Mian (1995). Collected sample was put inside tagged zipper bag and was rapidly bought to Plant Protection Laboratory of Agrotechnology Discipline, Khulna University, Khulna. Disease leaf was thoroughly washed under running tap water. Then the leafs were cut into convenient size (about 5 mm) where healthy and diseased tissues remain together. The cut leaf pieces were first thoroughly washed by sterilized distilled water and then transferred to 0.1% sodium hypocloride (NaOCl) solution and kept there for one minutes. Then those were washed with distilled water for three times. Excess surface water was absorbed with sterilized blotting paper. Excess NaOCl was removed by soaking with sterilized blotting paper. The cut pieces were then placed onto sterilized Potato Dextrose Agar (PDA) medium in glass petridishes with the help of sterilized forceps. All works were done in an aseptic condition. Led of the petri-dishes was sealed with Paraffin tape. Those were kept in the growth chamber at temperature of 25

±1°C for few days. Regular visual inspection was done to observe the proper growth of the colony. Contaminated cultures were removed immediately after detection. After incubation, temporary slide was made from the each culture plate and shown the mycelium growth under a dissecting stereo-zoom microscope (25 X) (Carl Zeiss, Germany).

3.5 Pathogenicity test

Pathogenicity of *Pestalotiopsis microspora* was tested by fresh whole leaves. The excised leaves were surface disinfected by dipping in 70% ethanol for 10 second. Then excess ethanol was absorbed with a sterilized blotting paper and adsorbed ethanol was washed for three times with distilled water for 3 times. Then leaves were placed onto moistened blotting paper in a petridish. Leaf was inoculated with drops of *Pestalotiopsis microspora* conidial suspension and incubated at 25 ± 1°C for appearance of symptoms (Djingra and Sinclair, 1986). 2 µl sterilized water was applied in a petri dish as control (Djingra and Sinclair, 1986)

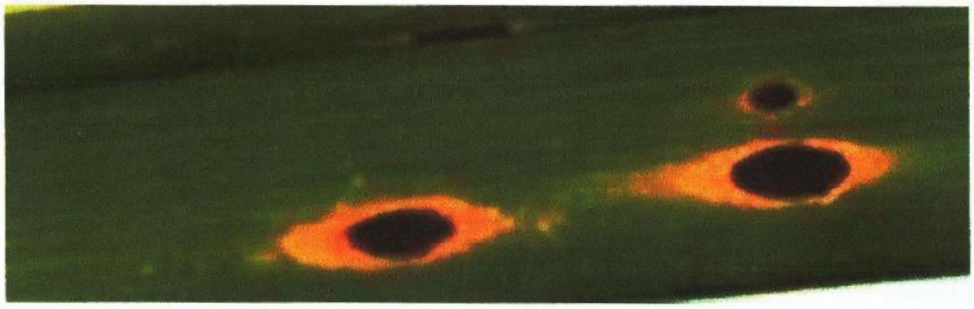


Figure 6. Pathogenicity test of coconut leaf spot

3.6 Experiment 1: Molecular Identification of *Pestalotiopsis microspora* isolates isolated from leaf spot of coconut leaf collected from different region of Khulna division

3.6.1 Morphological identification

Characteristics features of the germinated spore were observed cautiously by preparing temporary slides with glycerin solution. Morphological descriptions given by Jeewon et al.

(2003) were followed to identify *Pestalotiopsis microspora* conidia. Carl Zeiss compound microscope (40X) was used in this process (Carl Zeiss, Germany).

3.6.2 Molecular identification based on internal transcribed spacers (ITS)

Pestalotiopsis microspora isolates were grown in 250 mL conical flask which containing 100 mL potato dextrose broth [20% potato, 2% dextrose (w)} for 5 days. Cultures were incubated at 25±1 °C maintaining 12/12 h light and dark period. With the help of a rotary shaker (Wisd® laboratory instruments, Ireland) the flasks were shaken continuously at 150 rpm and approximately 20 g mycelia were harvested. Mycelium was separated by filtration through Whatman No.1 filter paper and washed thrice with sterile water. Then it was freeze dried at -86 °C in Ultra low freezer (Thermo scientific, USA) for lyophilizing the sample. The mycelial mass was finely ground in liquid nitrogen and 200 mg of mycelial powder was taken in sterile 1.5 mL eppendorf tube. PureLink fungal DNA purification kit (Invitrogen by life technologies, USA) was used for DNA extraction as per the manufacturer protocol. Genomic DNA of 13 isolates were extracted and stored at -20 °C and DNA extracts were run by electrophoresis in 1% agarose gel. This procedure was done to confirming the DNA content. Sspectrophotometer (Multiskan GO Microplate, Thermo Fisher Scientific, Germany) was used to quantify the purified DNA at 260 nm wave length. For PCR analysis the final concentration of the template DNA was adjusted at 50 ng µL. Template DNA was stored at -20 °C.

For molecular identification the isolates were selected. Internal transcribed spacers regions from the fungal isolates were amplified following polymerase chain reactions. Polymerase chain reactions were carried out in a 25 µL volume consisting of the following components in 0.2 mL thin walled PCR tubes for each isolate.

Table 2. Composition of Polymerase chain reaction chemicals for the molecular identification

SL No.	PCR reaction chemicals	Concentration	Quantity (µl)
1	Genomic DNA	50 ng μL^{-1}	1.0
2	Reaction buffer without $MgCl_2$	10X	2.5
3	dNTPs	10mM	0.5
4	Universal eukaryotic forward primer ITS5 GGAAGTAAAAGTCGTAACAAGG	5 pM μL^{-1}	0.5
5	Universal eukaryotic reverse primer ITS4 TCCTCCGCTTATTGATATGC	5 pM μL^{-1}	0.5
6	Tag DNA polymerase	5 U μL^{-1}	0.25
7	$MgCl_2$	25 mM	1.5
8	Molecular water (Sigma Aldrich, USA)		18.25
	Total volume of reaction mixture		25.0

3.6.3 Amplification Conditions

Amplification was carried out in programmable thermo-cycler (Biometra, Germany). The amplification programme was optimized which involved the following steps for successful amplification of PCR products

Table 3. Thermal Profile for ITS region sequencing

Thermal profile		Motives
Temperature (°C)	Duration (minutes)	
94.0	02	Hot start
95.0	0.5	Initial denaturation
55.0	01	Primer annealing
72.0	01	Primer extension
Repeated for 40 cycles		
72.0	05	Final extension
4.0	-	Hold

3.6.4 Gel documentation of PCR product

The amplified products were resolved by electrophoresis on 1% agarose gel stained with ethidium bromide (at 0.5 µg mL^{-1}) run at 60 volts in Tris Acetate EDTA buffer (1.0x TAE) for one hour. Resulted amplicon profiles of all the isolates across the primers were visualized under UV Tech Gel documentation System with computer program (Biometra gel documentation, A Biodoc Analyze 2.2 version, Germany). A molecular weight marker 1 kbp (Direct load, Sigma Aldrich, USA) was used for sizing of the amplicons.

3.6.5 ITS DNA sequence analysis

DNA sequencing of PCR products was done by the dideoxynucleotide chain termination method (Sanger et al., 1977). The rDNA homology searches were performed using the Basic Local Alignment Search Tool (BLAST) program through the internet server at the National Center for Biotechnology information (National institutes of Health, Bethesda, USA). Sequences and accession numbers for compered isolates were retrieved from the genbank database.

3.7 Experiment 2: Genetic Variability among the isolates of *Pestalotiopsis microspora* using RAPD marker

3.7.1 Experimental site and experimental details

The Experiment was performed in Plant Protection Laboratory and Horticulture Laboratory of Agrotechnology Discipline, Khulna University, Khulna, Bangladesh; from January 2021 to January 2022.

3.7.2 Collection of mycelium

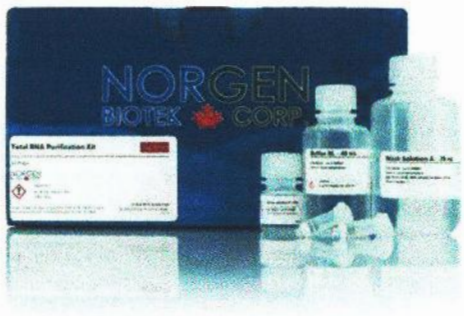
The presence of conidia of *Pestalotiopsis microspora* was observed by microscopy. To obtain a pure culture of *Pestalotiopsis microspora* a portion of the subculture was transferred aseptically to a PDA petridish. After that the petridish was sealed with the paraffin to avoid contamination. After that, the dish was incubated in an incubator at a temperature of 25±1°C for 5 days. Pure cultures were grown for 5 days. After the white cotton-like mycelium was produced on the Petri dish, we need to collect the mycelium.

3.7.3 Grinding of scrapped mycelium

50mg of mycelium was scrapped with a sterilized scalpel and scrapped materials were powdered with liquid nitrogen with sterilized morter-pistle and were taken in 1.5 ml eppendorf tube. Then those were stored in -86°C ultra-low freezer (Thermo Scientific, USA).

3.7.4 DNA isolation

NORGEN fungal DNA extraction kit (BIOTEK CROP) was used for DNA isolation according to the manufacturer's protocol.



Source: (https://www.google.com/search?q=NORGEN+fungal+DNA+extraction+kit)

Figure 7. Fungal DNA extraction kit

3.7.5 Storage of DNA

Genomic DNA of 13 isolates was extracted and DNA solution containing eppendorf tube was stored at -20°C. To confirm the presence of DNA materials test electrophoresis in 1% agarose gel was run. Picture of DNA residues was seen under UV light in Gel documentation system.

Figure 8. Eppendorf tubes containing isolated fungal DNA

3.7.6 DNA quantifications

Multiskn GO Microplate Spectrophotometer (Thermo Fisher Scientific, Germany) was used to quantify purified DNA at a wave length of 260 nm. The final concentration of the template DNA was adjusted to 50 ng $(\mu L)^{-1}$ for PCR and stored at -20°C.

3.7.7 Primer list

Seven Primers were used to find out the DNA banding pattern

Table 4. Primers and their corresponding sequence

SL No.	Primer Code	Primer Sequence (5'-3')
1	OPA-05	AGGGGTCTTG
2	OPA-09	GGGTAACGCC
3	OPA-10	GTGACGTAGG
4	OPB-01	GTTTCGCTCC
5	OPF-10	GGAAGCTTGG
6	OPA-18	AGGTGACCGT
7	OPA-03	AGTCAGCCAC

3.7.8 TAE buffer preparation

At first 250 ml 50X TAE buffer was prepared. In this case 60.5 g Tris base, 4.75 g EDTA salt was weighted with a digital balance. Then EDTA salt was mixed with 100 ml sterilized Mili-Q water with a magnetic hot stirrer. After getting clean disodium EDTA solution, 60.5 g tris base was added and mixed. Then 14.5 ml glacial acetic acid was added. Volume was adjusted to 250 ml with sterilized distilled water (Brown and Lorenz, 2016). Finally 980 ml sterilized Mili-Q water and 20 ml 50X TAE buffer was mixed to prepare 1X TAE buffer (Rebecca et al., 2011).

Figure 9. 1 X and 50 X TAE buffer

3.7.9 PCR amplification

Polymerase Chain Reaction was performed in a 0.2 mL thin walled PCR tube. Amplifications were performed in thermos cycler (Biometra 4, Germany) using the following protocols: 94°C for 4 minutes, followed by 40 cycles of 94°C for 30 seconds, 30°C for 1 minute and final extension at 72°C for 5 minutes. Then cool downed to 4°C.

Table 5. Polymerase chain reaction chemicals for the molecular characterization

SL No.	PCR reaction Chemicals	Concentration	Quantity (µL)
1.	Genomic DNA	50 ng μL^{-1}	5.0
2.	Reaction buffer without $MgCl_2$	10 X	1.0
3.	dNTPs	10 mN	0.2
4.	Tag DNA polymerase	5 U µL-1	0.2
5.	$MgCl_2$	25mN	1.2
6.	Molecular water (Sigma Aldrich, USA)	18 M	15.4
7.	RAPD primer	100 M	1.0
	Total volume of reaction mixture= 24.00		

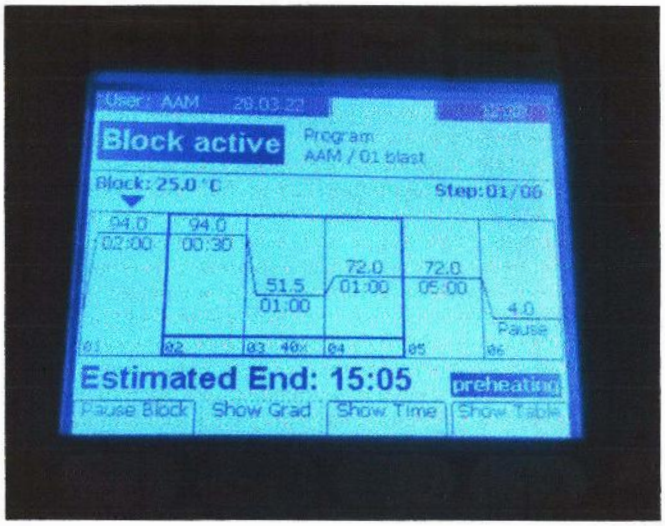


Figure 10. Image of PCR machine display (During work)

3.7.10 Agarose gel electrophoresis

Amplified products were separated by electrophoresis on 1% agarose gels with TAE running buffer and stained with ethidium bromide (at 0.5 µg mL^{-1}) run at 60 volts in Tris Acetate

EDTA buffer solution (1.0 X TAE) for one hour. High voltage switch option was pressed to supply electricity. Bioneer gel electrophoresis machine was used.

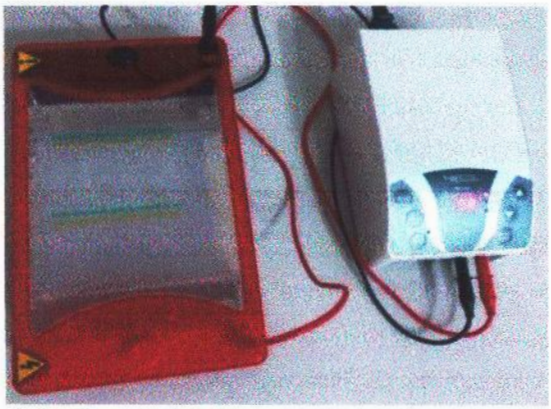


Figure 11. Image of gel run during work.

3.7.11 Gel documentation

The electrophoresis gels were scanned and photographs were taken with Biodoc Analyze 2.2 version computer program attached in gel documentation system (Biometra gel documentation, A Biodoc Analyse 2.2 version, Germany). A molecular weight marker 1kbp (Direct load, Sigma Aldrich, USA) was used for sizing of the amplicons.

3.7.12 PCR product analysis

Using the gel documentation system, RAPD-PCR bands were detected. For their presence (1) or absence (0), bands were scored. All scored band was considered as single allele/locus. Sizes of the bands were determined using standard 1kb ladder/marker. Six RAPD primers were used to produce band patterns among them four primers showed clear banding patterns. Some parameters such as polymorphism percentage, polymorphic information content, resolving power of the marker and marker index were calculated to check the in formativeness and discriminatory power of RAPD primers used in this study. Then binary data were subjected to similarity correlation analysis by sample matching coefficient (Sokal

and Michener, 1958) and then a UPGMA dendrogram was constructed by cluster analysis. Further Principal Component Analysis was performed to calculate eigenvalue and eigenvector then a 3D plot was made using NTSYSpc (Numerical Taxonomy and Multivariate Analysis System version 2.01e) portable software (Rohlf, 2002).

3.8 Calculation of Polymorphism percentage

Polymorphism percentage was calculated by dividing the number of polymorphic bands by the total number of bands and multiplied by 100.

$$\textbf{Polymorphism percentage} = \frac{\textbf{Number of polymorphic bands}}{\textbf{Total number of bands}} \times 100$$

Chapter IV

Results and Discussion

4.1 Experiment 1: Identification of *Pestalotiopsis microspora* isolates isolated from leaf spot of coconut leaf collected from different regions of Khulna division

The experiment was conducted in Plant Protection Laboratory of Agrotechnology Discipline at Khulna University, Khulna. Coconut leaf infected with grey leaf spot was collected from different locations of Khulna division. All the isolates were purified following single spore purification technique and finally found a total of 13 purified isolates after identification of *Pestalotiopsis microspora*. In pathogenicity test all the isolates showed pathogenic reaction. Finally a total 13 purified and pathogenic isolates of *Pestalotiopsis microspora* were preserved in test-tube slant with PDA medium at 4 °C in refrigerator (Figure 12)

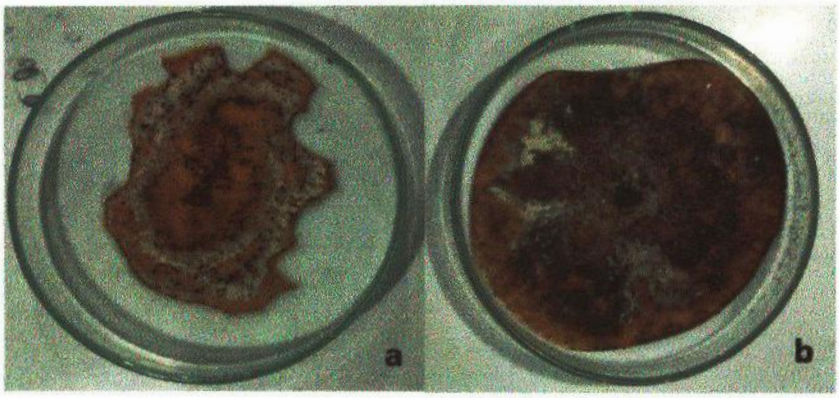


Figure 12. Purified Culture of *Pestalotiopsis microspora*

Identification of all 13 *Pestalotiopsis microspora* isolates was done based on their morphological and conidial characteristics (Figure 13) (Jeewon et al., 2003).

Figure 13. Showing conidia of *Pestalotiopsis microspora*

*M: Ladder, 1: Pm1, 2: Pm2, 3: Pm3, 4: Pm4, 5: Pm5, 6: Pm6, 7: Pm7, 8: Pm8, 9:Pm9, 10: Pm10, 11:Pm11, 12: Pm12, 13: Pm13

Figure 14. PCR amplification of ITS regions of tested isolates

For molecular identification from 13 isolates three isolates like Pm2, Pm8 and Pm11 isolates were randomly selected. Internal transcribed spacers (ITS) rDNA region of tested Pm2, Pm8 and Pm11 isolates were ranged respectively 593, 591 and 558. Their extent of similarity was 100% for Pm2 and Pm8 and near about 100% for Pm11. *Pestalotiopsis microspora* fungi matches with GenBank references are OK254035.1, OK254041.1 and AY681483.1.

Table 6. Molecular identification of *Pestalotiopsis microspora* isolates based on Matched GenBank accession number

Sl. No.	**Sample Code**	**ITS rDNA Region (bp)**	**Extent of the Similarity (%)**	**Sequenced ITS rDNA matched with GenBank ID**
1	Pm2	593	100%	*Pestalotiopsis microspora* (OK254035.1)
2	Pm8	591	100%	*Pestalotiopsis microspora* (AY681483.1)
3	Pm11	558	99.65%	*Pestalotiopsis microspora* (OK254041.1)

Table 7. Internal transcribed spacer rDNA sequence of selected *Pestalotiopsis microspora* isolates

Species name	ITS rDNA Sequence
Pestalotiopsis microspore Isolate Pm-2 Gene bank accession number **(OK254035.1)**	5'AGTCTCGTTGGTGACCAGCGGAGGGATCATTATAGAGTTTTCTAAACTCCCAACCCATGT GAACTTACCTTTTGTTGCCTCGGCAGAAGTTATAGGTCTTCTTATAGCTGCTGCCGGTGGA CCATTAAACTCTTGTTATTTTATGTAATCTGAGCGTCTTATTTTAATAAGTCAAAACTTTCA ACAACGGATCTCTTGGTTCTGGCATTTGGTGACAGCGGAGGGATCATTATAGAGTTTTCTA AACTCCCAACCCATGTGAACTTACCTTTTGTTGCCTCGGCAGAAGTTATAGGTCTTCTTAT AGCTGCTGCCGGTGGACCATTAAACTCTTGTTATTTTATGTAATCTGAGCGTCTTATTTTAA TAATCAAACTTTCACAACGGAGCTCTTGGGTCTCGTCCTACCTGATCCGAGGTCACCACAA AAAATTGGGGGTTTAGCGGCTGGGAGTATAGCACCTAACAAAAGCGAGAAAAAAATTAC TACGCTCAGAGGATACTACAAATCCGCCGTTGTATTTCGGAACTACACTATAAGAATATAT TCCCAACACTAAGCTAGGCTTAAGGGTTGAAATGACGCTCGAACA3'
Pestalotiopsis microspore Isolate Pm-8 Gene bank accession number **(AY681483.1)**	5'GTGGTGAACCAGCGGAGGGATCATTATAGAGTTTTCTAAACTCCCAACCCATGTGAACT TACCATTGTTGCCTCGGCAGAAGCTGCTCGGTGCACCCTACCTTGGAACGGCCTACCCTGT AGCGCCTTACCCTGGAACGGCTTACCCTGCAACGGCTGCCGGTGGACTACCAAACTCTTGT TATTTTATGGTTATCTGAGCGTCTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTT GGTTCTGGCATCGATGAAGAACGCAGCTGACCAGCGGAGGGATCATTATAGAGTTTTCTA AACTCCCAACCCATGTGAACTTACCATTGTTGCCTCGGCAGAAGCTGCTCGGTGCACCCTA CCTTGGAACGGCCTACCCTGACGCCTACCCTGGAACGGCTTACCCTGCAACGGCTGCCGG TGGACTACCAAACTCTGTTATTTTATGGGTATTTCCTACCTGATCCGAGGTCAACCATTAA AAATTGGGGGGTTTAGCGGCTAAAGACGCGCGACTCCGTCAAAAGCGAGATAAAATTACT ACGCTCAGAGGATATCGCAGATCCGCCGTTGATTTCAGGAGCTACA3'
Pestalotiopsis microspora Isolate Pm-11 Gene bank accession number **(OK254041.1)**	5'GTGGTGACCAGCGGAGGGATCATTATAGAGTTTTCTAAACTCCCAACCCATGTGAACTT ACCTTTTGTTGCCTCGGCAGAAGTTATAGGTCTTCTTATAGCTGCTGCCGGTGGACCATTA AACTCTTGTTATTTTATGTAATCTGAGCGTCTTATTTTAATAAGTCAAAACTTTCAACAACG GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG CAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGGCTGCCGGTGGACTA CCAAACTCTGTTATTTTATGGGTATTTCCTACCTGATCCGAGGTCAACCATTAAAAATTGG GGGGTTTAGCGGCTAAAGACGCGCGGATTGGTGACCAGCGGAGGGATCATTATAGAGTTT TCTAAACTCCCAACCCATGTGAACTTACCTTTGTCCCGCAGAAGTTCCTACCTGATCCGAG GTCACCACTAAAATTGGGGGTTTACGGCTGGGAGGGGCGCTCGGCAACGAGATATTCTAC 3'

4.1.2 Discussion

Pestalotiopsis microspora was also identified based on ribosomal DNA (ITS region) sequences following molecular identification method. Among thirteen isolates, three isolates like Pm2, Pm8 and Pm11 of 13 isolates were randomly selected for molecular identification. Internal transcribed spacers (ITS) region of selected three isolates was amplified and sequenced. ITS rDNA region of tested 3 isolates ranged from 558 bp to 593 bp (Table 6). The ITS rDNA sequences of tested isolates search result are presented in Table 6 and 7. Whereas, species level identification searches in the NCBI GenBank was successful by matching 100% with the *Pestalotiopsis microspora*. Therefore, all tested isolates were confirmed as *Pestalotiopsis microspora* according to the closest NCBI GenBank. Molecular identification is needed to confirm *Pestalotiopsis microspora* at the species level. rDNA- ITS have a unique potential for providing information about organisms. DNA sequencing of the ITS region has greatly increased the credibility of fungal identification. In the present investigation, the ITS region of the pathogenic fungi was sequenced and the tested isolates were identified as *Pestalotiopsis microspora*. Lacap et al. (2003); Promputtha et al. (2005) described that sequence analysis of the Internal Transcribed Spacer (ITS) region of nuclear ribosomal DNA (rDNA) has been widely used for molecular identification and phylogenetic diversity studies of fungi. In this study, isolated fungi were showed 100% similarity with *Pestalotiopsis microspora* in the GenBank. Results of this study reveal that experimental fungi matches with *Pestalotiopsis microspora* following GenBank references are OK254035.1, OK254041.1 and AY681483.1. The sequenced strain of fungi is named as *Pestalotiopsis microspora.* Vogler and Bruns (1998) noted that relatively rapid DNA sequence changes in the ITS region of rDNA genes can be used to determine inter-species variation.

4.2 Experiment 2: Genetic variability of *Pestalotiopsis microspora* isolates by RAPD method

The experiment was conducted in Plant Protection Laboratory of Agrotechnology Discipline at Khulna University, Khulna. Coconut leaf infected with leaf spot was collected from different locations of Khulna division. Pure culture of *Pestalotiopsis microspora* was prepared. Then mycelium was harvested for DNA isolation. Seven RAPD primers were used to produce band patterns among them four primers showed clear banding patterns.

4.2.1 Parameters used for evaluation of polymorphism in RAPD marker

Polymorphism percent (Table 8) was 100% for OPA-05, OPA-09, OPA-10, OPB-01, OPF-10 OPA-03 primers and 80% for OPA-18. Average polymorphic percentage 97.14. Different polymorphism percent show efficiency of each marker to separate and segregate in studied genotypes.

Table 8. Parameters used for evaluation of marker effectiveness

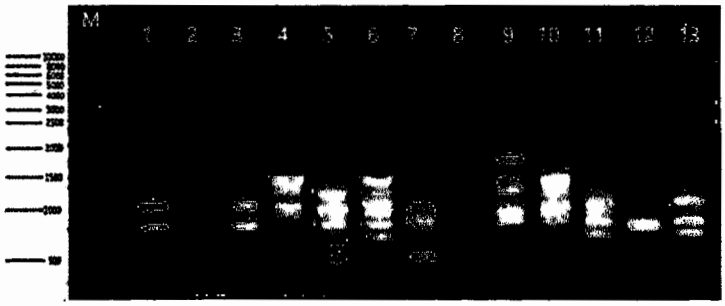
Name Code	Primer Sequence (5' to 3')	GCC%	MNAB	NPB	NMB	PP (%)
OPA-05	AGGGGTCTTG	60	7	7	0	100
OPA-09	GGGTAACGCC	70	6	6	0	100
OPA-10	GTGACGTAGG	60	6	5	1	100
OPB-01	GTTTCGCTCC	60	6	6	0	100
OPF-10	GGAAGCTTGG	60	5	5	0	100
OPA-18	AGGTGACCGT	60	5	5	0	80
OPA-03	AGTCAGCCAC	60	5	5	0	100
Average polymorphic percentage 97.14						

*GCC: Guanine-Cytosine Content, MNAB: Maximum Number of Amplified Band, NBP: Number of Polymorphic Band, MBP: Number of Monomorphic Band; PP: Polymorphic Percentage

4.2.2 Determination of Variability based on amplified DNA banding pattern

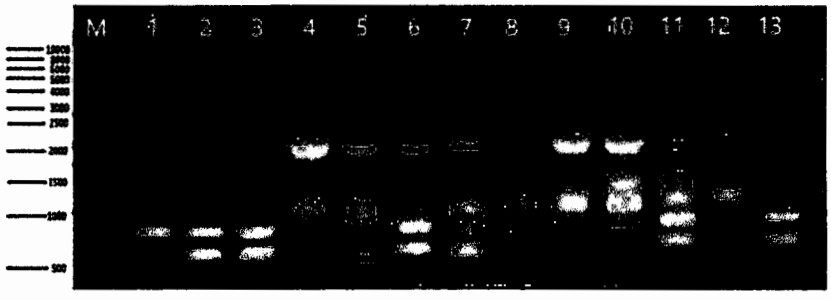
Two types of bands were found in the amplified DNA products. Monomorphic and polymorphic, the bands existing in all individuals are monomorphic. On the other hand, those bands present in one or more individuals, but not in all individuals are polymorphic bands (Mehetre et al., 2004).

A total of 40 DNA bands from all the studied primers were obtained after RAPD analysis. Among them, 39 were polymorphic and 1 was bands of monomorphic. After completion of the experiment, 80% to 100% polymorphism was observed. Nevertheless, considering seven primers, the average number of bands was 40. The amplified DNA fragments ranged from 500 to 2500 bp for all primers (Figure 15a to 15g).



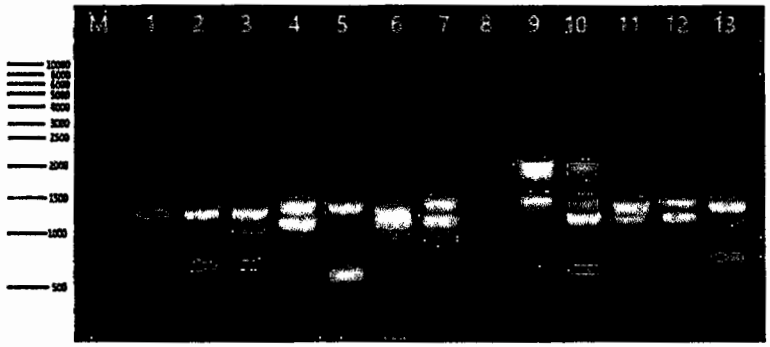
*M: Ladder, 1: Pm1, 2: Pm2, 3: Pm3, 4: Pm4, 5: Pm5, 6: Pm6, 7: Pm7, 8: Pm8, 9:Pm9, 10: Pm10, 11:Pm11, 12: Pm12, 13: Pm13

Figure 15a. RAPD bands generated by primer OPA-05 for *Pestalotiopsis microspora* isolated from coconut leaves



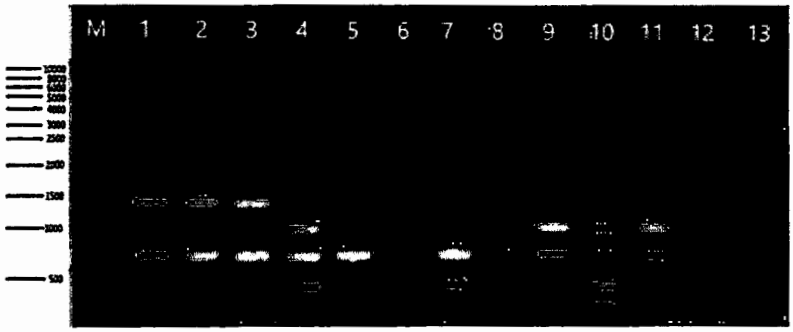
* M: Ladder, 1: Pm1, 2: Pm2, 3: Pm3, 4: Pm4, 5: Pm5, 6: Pm6, 7: Pm7, 8: Pm8, 9:Pm9, 10: Pm10, 11:Pm11, 12: Pm12, 13: Pm13

Figure 15b. RAPD bands generated by primer OPA-09 for *Pestalotiopsis microspora* isolated from coconut leaves



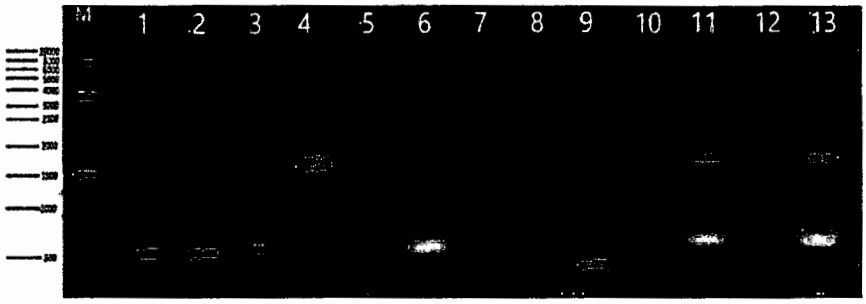
*M: Ladder, 1: Pm1, 2: Pm2, 3: Pm3, 4: Pm4, 5: Pm5, 6: Pm6, 7: Pm7, 8: Pm8, 9:Pm9, 10: Pm10, 11:Pm11, 12: Pm12, 13: Pm13

Figure 15c. RAPD bands generated by primer OPA-10 for *Pestalotiopsis microspora* isolated from coconut leaves



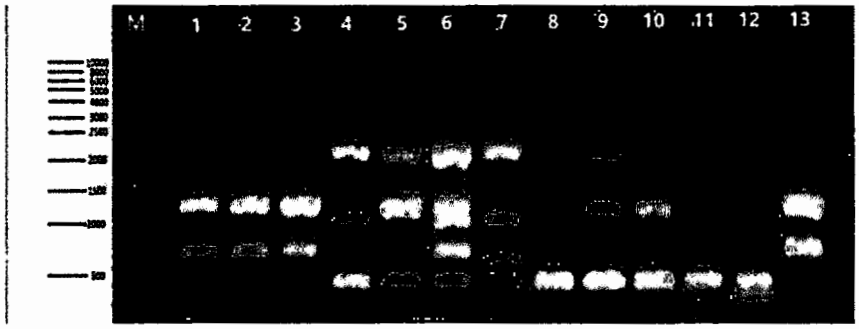
*M: Ladder, 1: Pm1, 2: Pm2, 3: Pm3, 4: Pm4, 5: Pm5, 6: Pm6, 7: Pm7, 8: Pm8, 9:Pm9, 10: Pm10, 11:Pm11, 12: Pm12, 13: Pm13

Figure 15d. RAPD bands generated by primer OPB-01 for *Pestalotiopsis microspora* isolated from coconut leaves



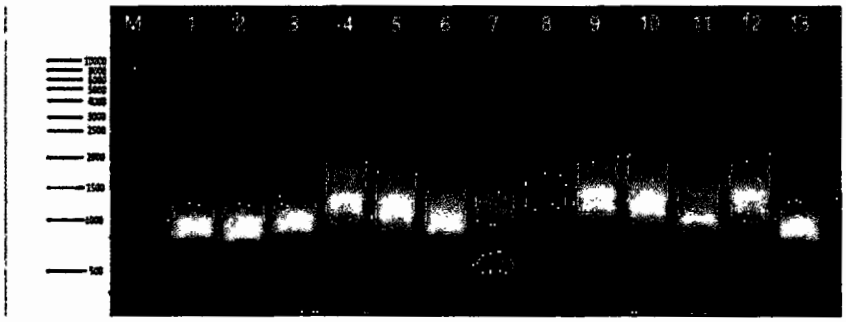
*M: Ladder, 1: Pm1, 2: Pm2, 3: Pm3, 4: Pm4, 5: Pm5, 6: Pm6, 7: Pm7, 8: Pm8, 9:Pm9, 10: Pm10, 11:Pm11, 12: Pm12, 13: Pm13

Figure 15e. RAPD bands generated by primer OPF-10 for *Pestalotiopsis microspora* isolated from coconut leaves



*M: Ladder, 1: Pm1, 2: Pm2, 3: Pm3, 4: Pm4, 5: Pm5, 6: Pm6, 7: Pm7, 8: Pm8, 9:Pm9, 10: Pm10, 11:Pm11, 12: Pm12, 13: Pm13

Figure 15f. RAPD bands generated by primer OPA-18 for *Pestalotiopsis microspora* isolated from coconut leaves



*M: Ladder, 1: Pm1, 2: Pm2, 3: Pm3, 4: Pm4, 5: Pm5, 6: Pm6, 7: Pm7, 8: Pm8, 9:Pm9, 10: Pm10, 11:Pm11, 12: Pm12, 13: Pm13

Figure 15g. RAPD bands generated by primer OPA-01 for *Pestalotiopsis microspora* isolated from coconut leaves

4.2.4 Similarity correlation matrix

The correlation of genetic similarity among the thirteen (13) isolates of *Pestalotiopsis microspora* was shown in the Table No. 9. Genetic similarity of 89% was observed between Pm2 and Pm3, which were collected from Rupsha stand road, Rupsha and Itola, Dumuria in Khulna region. The lowest genetic similarity of only 12% was observed between Pm9 and Pm13 (the maximum genetic difference was 88%), which were collected from Koia bazar, Harintana and Batiaghata, Dumuria in Khulna region.

Table 9. Genetic similarity correlation matrix among 13 isolates of *Pestalotiopsis microspora*

Isolate No.	Pm1	Pm2	Pm3	Pm4	Pm5	Pm6	Pm7	Pm8	Pm9	Pm10	Pm11	Pm12	Pm13
Pm1	-												
Pm2	0.82	-											
Pm3	0.83	0.89	-										
Pm4	0.23	0.23	0.25	-									
Pm5	0.33	0.37	0.34	0.48	-								
Pm6	0.41	0.46	0.42	0.46	0.61	-							
Pm7	0.42	0.44	0.41	0.52	0.58	0.63	-						
Pm8	0.19	0.17	0.16	0.32	0.45	0.25	0.29	-					
Pm9	0.22	0.21	0.24	0.61	0.46	0.31	0.43	0.36	-				
Pm10	0.26	0.26	0.27	0.72	0.53	0.38	0.54	0.39	0.75	-			
Pm11	0.52	0.56	0.57	0.29	0.46	0.51	0.48	0.32	0.40	0.42	-		
Pm12	0.29	0.33	0.30	0.43	0.48	0.44	0.56	0.42	0.44	0.47	0.42	-	
Pm13	0.56	0.71	0.63	0.16	0.31	0.45	0.32	0.22	0.12	0.17	0.44	0.26	-

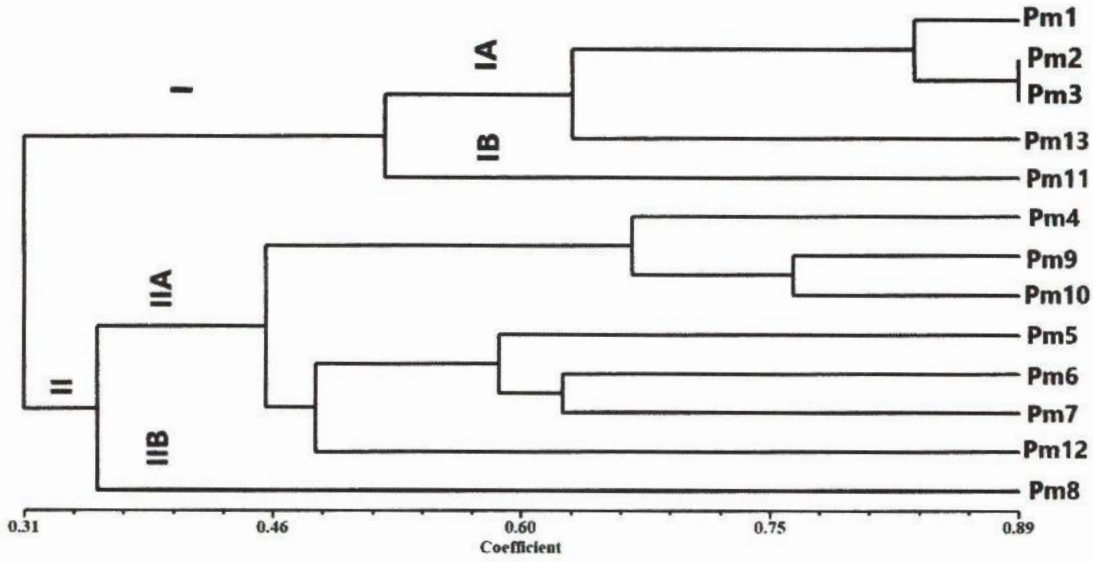


Figure 16. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram

4.2.5 Cluster analysis

A UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram (Figure 16) was constructed from the sample matching correlation matrix. Two distinct clusters were visible. Pm1, Pm2, Pm3, Pm11 and Pm13 was belonging in cluster I and cluster II was containing Pm4, Pm5, Pm6, Pm7, Pm8, Pm9, Pm10 and Pm12 isolates. Cluster I was divided into two sub-clusters. Pm11 was in IB sub-cluster which was collected from Noapara, upazilla of Jashore district under Khulna division. Pm1, Pm2, Pm3 and Pm13 isolates belonged to IA sub-cluster. Cluster II was further divided into two sub-clusters where Pm8 isolates belonged to sub-cluster II B which was collected from Narikel tola of shatkhira district under Khulna division and Pm4, Pm5, Pm6, Pm7, Pm9, Pm10 and Pm12 isolates belonged to sub-cluster II A.

4.2.6 Principal component analysis

PCA transforms a set of correlated variables to a new set of uncorrelated variables. The PCA was simply shows components which were close to the original variables but arranged in decreasing order of variance. The PCA mostly confirmed by the cluster analysis. In case of genetically similar isolates Pm2 and Pm3 isolates; which formed its own group in cluster analysis and PCA analysis (Figure 16 and Figure 17) gave the evidences. That means no genetically variation was found among the Pm2 and Pm3 isolates which was collected from Rupsha stand road, thana- Rupsha and Itola, Upailla- Dumuria under Khulna division. From this PCA analysis it also disclose that positive co-relation was belonging within the Pm1, Pm2, Pm3, Pm5, Pm6,Pm11, Pm12 and Pm13 isolates and negative co-relation was belonging among the Pm4, Pm5, Pm8, Pm9 and Pm10 isolates.

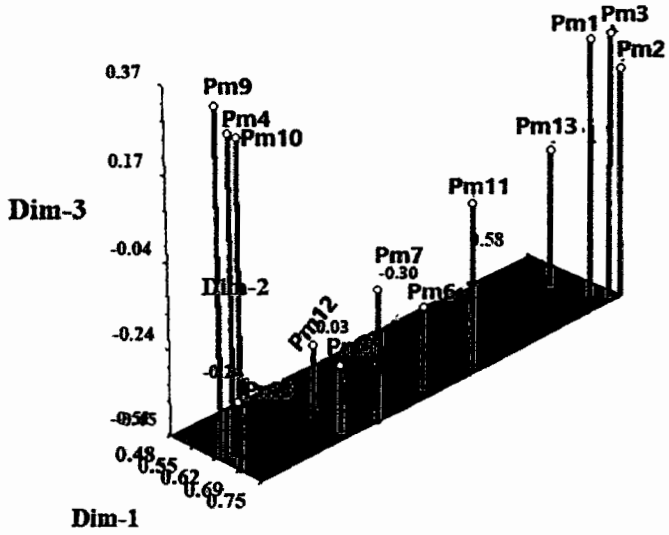


Figure 17. Three-dimensional plot of PCA showing relationships among 13 *Pestalotiopsis microspora* isolates using genetic traits

4.2.7 Discussion

Analysis of plant pathogen diversity has been revolutionized by molecular techniques. In particular, the PCR technique has contributed to the population structure and genetic diversity (Banerjee et al., 2014). In this experiment, genetic variation among isolates of *Pestalotiopsis microspora* was detected.

In the RAPD analysis, seven RAPD primers were used, namely OPA-05, OPA-09, OPA-10, OPB-01, OPF-10, OPA-18, OPA-03, showing clear resolution and finding genetic variability. Among all primers, primer OPA-05 showed more reproducible DNA bands for *Pestalotiopsis microspora*. A total of 40 amplified DNA bands were obtained by using seven primers. Among them, only one momomorphic band was observed after RAPD analysis. After completion of the experiment, 80% to 100% polymorphism was observed. Sarvottam et al. (2009) performed experiments with 10 RAPD primers with 1517 bands were amplified. All the bands were polymorphic, which correlates with the results of this study.

In this experiment, a maximum of 88% dissimilarity was found between Pm9 and Pm13 isolates. Pm9 and Pm13 isolates were collected from Koia bazar, Harintana and Batiaghata, Dumuria under Khulna division. From the findings *Pestalotiopsis microspora* 89% genetic similarity was observed between Pm2 and Pm3, which were collected from Rupsha stand road, Rupsha and Itola, Dumuria in Khulna region.

Sarvottam et al. (2009) experimented with 42 *Pestalotiopsis* isolates were collected from five different regions in southern India. Genetic diversity was studied from 22 isolates using RAPD marker. They were found only 8.8% of similarity between the 22 isolates by RAPD marker. From the findings of this experiment exposed highly genetically variability exists among the thirteen isolates.

Cluster analysis showed two major clusters. Pm1, Pm2, Pm3, Pm11 and Pm13 was belonging in cluster I and cluster II was containing Pm4, Pm5, Pm6, Pm7, Pm8, Pm9, Pm10, Pm12 isolates. Cluster I and II was further sub-divided into two sub cluster. Pm11 was in IB sub-cluster which was collected from Noapara, Jashore under Khulna division and Pm1 Pm2 Pm3 and Pm13 isolates belonged to IA sub-cluster. Where only Pm8 was in sub cluster II B which was collected from Narikel tola, Shatkhira sadar under Khulna division and Pm4, Pm5, Pm6, Pm7, Pm9, Pm10, Pm12 were in sub-cluster II A. Result of Sarvottam et al. (2009) disclose three clusters based on RAPD method among 19 isolates. From the present experiment two distinct clusters by observing 13 isolates of *Pestalotiopsis microspora*.

Wei et al. (2007) and Tejesvi et al. (2009) they reported that molecular diversity on the endophytic species of *Pestalotiopsis* was not largely influenced by geographical factors. From the findings, Pm9 and Pm13 were collected from different locations but they showed maximum genetic variability.

Again Liu et al. (2006); Wei et al. (2007) and Watanabe et al. (2010) described four new species and most genetic variability was found between *P. macrochaeta*, *P. uvicola* and *P. pallidotheae* which was relevant to the findings.

Chapter V

Summary and Conclusion

5.1 Summary

The experiment was conducted to detect molecular identification and genetic variation among thirteen *Pestalotiopsis microspora* isolates. Leaf spot infected plant materials were collected from different locations of Khulna division. Thirteen isolates of *Pestalotiopsis microspora* were isolated following standard procedure in Plant Protection Laboratory of Agrotechnology Discipline at Khulna University, Khulna. Mycelium was harvested for each isolate to obtained DNA by using DNA purification kit. For molecular identification from 13 isolates three isolates like Pm2, Pm8 and Pm11 isolates were randomly selected. The internal transcribed spacer (ITS) rDNA regions of the tested Pm2, Pm8 and Pm11 isolates ranged from 593, 591 and 558 respectively with 100% similarity for Pm2 and Pm8 and close to 100% for Pm11. All tested isolates were confirmed as *Pestalotiopsis microspora* according to the closest NCBI GenBank references are OK254035.1, OK254041.1 and AY681483.1.

For RAPD analysis, using seven RAPD primers namely OPA-05, OPA-09, OPA-10, OPB-01, OPF-10, OPA-18, OPA-03 and they showed clear resolution and identified genetic variability. Among all primers, primer OPA-05 showed more RAPD bands for *Pestalotiopsis microspora.* After the experiments were completed, 80% to 100% polymorphism was observed.

In this experiment, a maximum of 88% dissimilarity was found between Pm9 and Pm13 isolates. Pm9 and Pm13 isolates were collected from Koia bazar, Harintana and batiaghata, Dumuria under Khulna division. From the findings *Pestalotiopsis microspora* 89% genetic

similarity was observed between Pm2 and Pm3, which were collected from Rupsha stand road, Rupsha and Itola, Dumuria under Khulna division.

Two distinct clusters were visible. Pm1, Pm2, Pm3, Pm11 and Pm13 was belonging in cluster I and cluster II was containing Pm4, Pm5, Pm6, Pm7, Pm8, Pm9, Pm10 and Pm12 isolates. Cluster I was divided into two sub-clusters. The highest genetic similarity was found on cluster IA between Pm2 and Pm3 collected from different locations. Pm11 was in IB sub-cluster which was collected from Noapara of Jashore district under Khulna division. Pm1, Pm2, Pm3 and Pm13 isolates belonged to IA sub-cluster. Cluster II was further divided into two sub-clusters where Pm8 isolates belonged to sub-cluster II B which was collected from Narikel tola of shatkhira district under Khulna division and Pm4, Pm5, Pm6, Pm7, Pm9, Pm10 and Pm12 isolates belonged to sub-cluster II A. There is a certain degree of genetic variability present among *Pestalotiopsis microspora* species.

The PCA mostly confirmed by the cluster analysis. In the case of genetically similar isolates Pm2 and Pm3 isolates, they formed their own groups in cluster analysis and PCA analysis. That means no genetically variation was found among the Pm2 and Pm3 isolates which was collected from Rupsha stand road, Rupsha and Itola, Dumuria under Khulna division. From this PCA analysis it also disclose that positive co-relation was belonging within the Pm1, Pm2, Pm3, Pm5, Pm6,Pm11, Pm12 and Pm13 isolates and negative co-relation was belonging among the Pm4, Pm5, Pm8, Pm9 and Pm10 isolates.

5.2 Conclusion

1. Three isolates such as Pm2, Pm8 and Pm11 isolates were randomly selected for internal transcribed spacer (ITS) rDNA region testing. Based on the closest NCBI GenBank reference, 100% similarity was confirmed for *Pestalotiopsis microspora.*

2. In the RAPD analysis, 97.14% polymorphism was confirmed using seven RAPD primers.
3. Among thirteen isolates maximum 88% dissimilarity was found between Pm9 and Pm13 and they showed significantly more genetic variability.
4. UPGMA dendrogram showed two distinct clusters. Pm1, Pm2, Pm3, Pm11 and Pm13 was belonging in cluster I and cluster II was containing Pm4, Pm5, Pm6, Pm7, Pm8, Pm9, Pm10, Pm12 isolates.
5. Isolates of *Pestalotiopsis microspora* originated from different localities, but they had high genetic variability.

CHAPTER V

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