GENETIC DIVERSITY ANALYSIS OF *Phytophthora colocasiae* USING SSR MARKERS

By

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(2012-09-107)

THESIS

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DECLARATION

I, hereby declare that this thesis entitled "**Genetic diversity analysis of *Phytophthora colocasiae* using SSR markers**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar title, of any other university or society.

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3

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Akshara George

Dedicated to My Parents & Brother

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVATIONS

%	Percentage
A_{230}	Absorbance at 230 nm wavelength
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of Molecular Variance
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C. esculenta	*Colocasia esculenta*
cm	centimetre
CTAB	Cetyltrimethyl ammonium bromide
cv.	Cultivar
d. a. i.	Days after inoculation
DMRT	Duncan's Multiple Range Test
DNA	Deoxyribonucleic acid
dNTPs	Deoxy nucleotide tri phosphates
E	East
EDTA	Ethylene diamine tetra acetic acid
EST	Expressed Sequence Tags
FAOSTAT	Food and Agriculture Organization Statistical Database
g	gram
h	Hour
ha	Hectare
ICAR-CTCRI	ICAR- Central Tuber Crops Research Institute

ITS	Internal Transcribed Spacer
kb	Kilo bases
kg	Kilogram
L	Litre
m	Meter
M	Molar
mg	milligram
$MgCl_2$	Magnesium Chloride
min	Minute
ml	Millilitre
mm	Millimeter
mM	Millimolar
N	North
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nm	Nanometer
°C	Degree Celsius
OD	Optical density
P. colocasiae	*Phytophthora colocasiae*
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PVP	Polyvinylpyrrolidine
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid

RNase	Ribonuclease
rpm	revolutions per minute
s	second
SDS	Sodium dodecyl sulphate
SNP	Single Nucleotide Polymorphism
sp.	Species
spp.	Species (plural)
SSR	Simple sequence repeat
Taq	*Thermusaquaticus*
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
T_m	Melting temperature
Tris HCl	Tris hydrochloride
U	Enzyme units
UPGMA	Unweighted pair group with Arithmetic average
UV	Ultra violet
V	Volt
v/v	volume/volume
viz.	Namely
W	Watt
w/v	weight/volume
Ypt	RAS related protein
µg	Microgram
µl	Microlitre
µM	Micromolar

INTRODUCTION

1. INTRODUCTION

Taro (*Colocasia esculenta* (L.) Schott) a member of *Araceae* family, is grown largely in humid tropical areas of the world. Taro acts as a vital food crop in the diets of many people around the world, and it is the fourteenth most dined vegetable globally (Lebot and Aradhya, 1991). Almost every parts of the crop, like corm, flowers, rhizome, leaves and stalk are consumable with adequate amount of starch. Taro is considered as a rich source of carbohydrates, minerals, proteins, vitamins and contain several medicinal properties to cure ulcers, tuberculosis, fungal infection and pulmonary congestion (Sharma *et al.*, 2008).

Corm rot and leaf blight, are the most destructive diseases of taro caused by the oomycete pathogen *Phytophthora colocasiae*. Leaf blight of taro has become a limiting factor for its production and results in a yield loss of up to 30–50 % (Thankappan, 1985; Jackson *et al.*, 1980; Misra and Chowdhury, 1997). The most frequent symptom of leaf blight is the appearance of water-soaked spots in the leaf lamina, which increase in number and size. With the disease advancement, lesions become irregular in shape, enlarge and color became dark brown with yellow margins. A characteristic feature of lesions is the formation of droplets of exudate in reddish-brown colour, oozing from both the lower and upper surface of water-soaked margins which looks like *sclerotia* when dried.

As *P. colocasiae* is a diploid heterothallic oomycete, it requires both mating types A1 and A2 for the oospores generation (Tyson and Fullerton, 2007). They produce thick-walled sexual oospores and thick-walled asexual chlamydospores for survival in the unfavorable environmental conditions. In addition, *Phytophthora*can produce asexual sporangia, which is dispersed by water and wind (Alexopoulos *et al.*, 1996). Under favorable weather conditions with frequent rains and temperature nearly 28°C, the disease appears to spreads quickly across the entire field area (Misra *et al.*, 2008).

Several approaches including crop rotation and the use of copper containing fungicides have been used to fight the disease. The presence of waxy coating on the leaf lamina of taro makes fungicides ineffective and uneconomical as repeated applications of huge quantity fungicides are required. Moreover, relying heavily on their use, can results in elevated frequency of the resistant mutants, particularly in populations of pathogen with the greater evolutionary potential and ability (McDonald and Linde, 2002).

Understanding the characteristics of pathogen populations are highly needed for designing proper disease management strategies. Molecular markers are efficient tools now a days used to track genotypes and useful to study pathogen diversity based on the DNA sequence polymorphisms. Diversity analysis of pathogen populations are vital to develop effective disease management practices. Genetic diversity can be referred as any variation in nucleotides, genes, chromosomes or whole genome of an organisms (Wang *et al.*, 2009).

Simple sequence repeats (SSR), also known as microsatellites, are widely used reliable genetic markers for diversity studies. As microsatellites are liable to mutations, they are highly informative with several advantages like low cost and PCR based easy detection. Microsatellites are widely distributed in the genome of eukaryotes and they are stretches of DNA sequences with mono-, di-, tri-, tetra- and penta tandemly repeated nucleotide units (Powell *et al.*, 1996). They are co-dominant with a higher level of polymorphism (Liu *et al.*, 1995). SSR are ideal DNA markers for genetic mapping and population study of pathogens, because of their abundance (Singh *et al.*, 2013). extensively microsatellites are used for the exploration of reproductive biology and genetic structure of several plant pathogens (Tenzer *et al.*, 1999), including *Phytophthora* (Dobrowolski *et al.*, 2003).

For the proper understanding of the pathogen epidemiology and host–pathogen coevolution, genetic diversity studies are very essential. Moreover, it is essential for initiating better breeding programs for the development of taro

cultivars resistant to pathogens and in screening this will help to identify duplicative isolates. It also enables the development of highly reliable and sensitive diagnostic tests for pathogen and such tests would be helpful in determining pathogen's geographical distribution. Hence the specific objectives of the work were formulated as:

1. To study the genetic diversity of *Phytophthora colocasiae* populations using SSR markers.
2. To study the morphological and molecular characterization of *Phytophthora colocasiae* isolates.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 ROOT AND TUBER CROPS

Root and tuber crops have long been cultivated in the tropical areas of the Asia, Pacific and Africa as source of dietary energy (Plowman, 1969). Tropical root and tuber crops including cassava, aroids, yam and sweet potato are consumed as staple or subsistence food crop especially in the developing countries (Ravi *et al.*, 1996; Lynch, 2015). The relative significance of these crops is obvious through their annual global production which is approximately 836 million tonnes with Asia as the main producer (FAOSTAT, 2013). While cereals provide much of the dietary energy for a large section of the global population, root crops are an ideal source of energy and they find a vital position in the food security of tribal populations. Even though the agronomic properties of tuber crops have been well studied and documented, their industrial quality, medicinal and nutritional properties have not been studied extensively.

The Western Ghats and North Eastern Himalayas which are the two global biodiversity hot spots, particularly rich in tuber crops and its wild relatives. South East Asia is considered as the Centre of origin of taro, a clonally propagated aroid (Kuruvilla and Singh, 1981). Effective conservation and sustainable utilization of plant biodiversity is indispensable for meeting the present and future requirements of tuber crops in India (Edison *et al.*, 2006).

2.2 TARO

Colocasia esculenta (L.) Schott commonly known as taro is a clonally propagated herbaceous plant of the monocotyledonous family *Araceae*. The crop was first cultivated in South East Asia, has later spread globally and is now considered as an important crop in Asia, Africa, Caribbean and Pacific (Rao *et al.*, 2010). It is the 14th most consumed vegetable with about 12 million tonnes production around the globe (Lebot and Aradhya 1991). Almost every parts of taro

including cormels, corm, stalk, rhizome, flowers and leaves are consumable (Lakhanpaul *et al.*, 2003). It is treated as a vital source of proteins, carbohydrates, vitamins and minerals (Misra and Sriram 2002) and has several medicinal properties to reduce ulcers, tuberculosis, fungal infection and pulmonary congestion (Sharma *et al.*, 2008). Moreover, taro corms are used in several manufacturing industries as raw materials for the production of alcohols and high fructose syrup (Misra *et al.*, 2008). These perspectives make taro as a significant and valuable tuber crop.

2.2.1 Taxonomy and genetics

Taro is an edible root crop of the monocotyledonous family *Araceae* within the *Colocasioideae* sub-family. According to the taxonomists, there exist two different botanical varieties of taro (Purseglove, 1972). One is the dasheen (*C. esculenta* var. esculenta), having a large cylindrical central corm with suckers, stolons, and a few generally not edible small cormels. The second botanical variety is the eddoe (*C. esculenta* var. antiquorum) having a smaller globular central corm surrounded by a large number of cormels arising from the corm, which are the main yield. The vital distinguishing characteristic between the two different botanical varieties is their inflorescence which has, the sterile appendage of the spadix is much longer in eddoe types (at least three times longer) than that of the dasheen. Majority of the taro cultivated in the Asia and Pacific regions is of the dasheen type (Purseglove, 1972). Globally there are numerous agronomic cultivars for taro grown mainly in humid tropical regions of the world. These can be distinguished on the basis of their agronomic characters, corm size or shape, shoot characteristics, or cormel characters (Coates *et al.*, 1988).

2.2.2 Origin and distribution

Ethno-botanical suggestions such as occurrence of wild cultivars of taro, states that they were originated in various locations of South Eastern Asia. From its center of origin, it is believed that taro spread to the Eastern parts of South East

Asia, and to Japan, the Pacific Islands and China. However, the largest cultivation and production area of taro is believed to be in West Africa (Purseglove, 1972).

2.2.3 Morphology and Anatomy

Taro is a succulent, herbaceous root crop that grows up to 1-2m in height, which consists of a central corm from which cormels, roots, stolons and the shoot arise. The central corm characterizes as the major stem structure of the crop in both the taro varieties *viz*., eddoe and dasheen. The surface of the corm is marked with several rings indicating the points of senesced leaves attachments. At the nodal positions on the corm, there exists axillary buds. The corm apex depicts the origin of growing point, and present near to the ground level. The only plant part that is visible above the ground is the growing leaves which appears in a whorl from the corm apex. Each taro leaf is composed of a big lamina and an erect petiole. The petiole is found to be thinner towards its attachment to the lamina and thickest at its base. The texture of petiole is spongy in its interior, and contain several air spaces which usually helps in gaseous exchange in flooded or swampy conditions. For several taro types, peltate leaf attachment was found in which the petiole is attached to the middle and not at the edge of the lamina. Thus tannia which is having a hastate leaf (the petiole is attached at the edge of the lamina) attachment is distinguished from taro which is having a peltate leaf attachment.

The root system of taro is fibrous and lies mostly in the top one meter of soil. The internal structure of corms, daughter corms and cormels are highly similar with a thick periderm as outer layer. Starch-filled ground parenchyma lies inside it. Ground tissue contain calcium oxalate bundles or raphides rich cells (Idioblasts). The itchiness or acridity of taro is due to the presence of these raphides.

Flowering in taro is occasional, and restricted to a few cultivers (generally diploids) under natural conditions. The inflorescence of taro usually arises from the axils, or from the center of unfolded leaves. A short peduncle, spathe and a spadix are the major components of inflorescence. The spadix which is a spike, containing a central axis with numerous attached sessile flowers. Six –fourteen cm long spadix, containing male flowers towards the tip, sterile flowers in between and

female flowers at its base. The spathe is a large bract and yellow in colour with 20 cm length, and it wraps the spadix. At the apex, the upper portion of the spadix is rolled inward. But it is open on one side to expose the male flowers. Fruits, usually when produced, appears at the bottom part of the spadix. Every fruit measures 3-5mm in diameter and contains numerous number of seeds. Each seed is a berry and has a hard testa. In addition to embryo, the seed contains an endosperm (Sharma *et al.*, 2008).

2.2.4 Nutritional value

The major economically important parts of the taro plant are corms, cormels, as well as leaves. Leaves of taro are frequently consumed as a vegetable and depicts an important source of vitamins, specifically folic acid. Taro leaves are rich source of minerals and in which potassium is abundant and iron is lacking. Onwueme *et al.* (1994) reported that the corms are vital energy source especially in the form of digested starch (13-29%). They are abundant in carbohydrates and low in proteins and fat (Wanasundera and Ravindran, 1994). Essential amino acids like phenylalanine and leucine are relatively abundant in taro corms (Onwueme *et al.*, 1994).

2.2.5 Diseases and pests

Taro cultivation has reduced over past 30 years due to pests and disease attacks. About 130 pests and diseases of taro have been recorded with influences ranging from moderate to lethal. Taro leaf blight and viral diseases are the most important constraints for taro cultivation as it causes severe reductions in the yield and ultimately plant destructions. Reduction in corm quality and size, with a 20% yield loss being reported as a result of viral infections in taro. Presently it has been reported that 5 viruses infect taro with varying distribution in taro growing regions *ie, Dasheen mosaic virus* (DsMV), *Colocasiae bobone disease virus* (CBDV), *Taro bacilliform virus* (TaBV), *Taro reo virus* (TaRV) and *Taro vein chlorosis virus* (TaVCV) (Revill *et al.*, 2005). Research programmes dealing with taro mainly aims to combat factors that can ruin taro production.

2.3 TARO LEAF BLIGHT

It is believed that taro is affected by more than 10 serious pests and diseases in different taro growing locations of the world (Kohler *et al.*, 1997; Singh *et al.*, 2012). Of the different types of taro diseases, taro leaf blight (TLB) caused by the oomycete pathogen *Phytophthora colocasiae* is of vital importance since it causes 50% yield reduction (Jackson *et al.* 1980; Thankappan 1985; Misra and Chowdhury 1997). It was first reported by Raciborski from Java in 1900 and in 1913, Butler and Kulkarni reported leaf blight of taro for the first time in India. Extensive foliar damage is the characteristic feature of this disease and it causes damages to leaves and petioles of infected taro plant. The disease quickly spreads across entire fields and results in extensive yield loss, under cloudy weather conditions having a temperature of 28 ^{0}C with intermittent rains (Trujillo 1965; Thankappan 1985). This pathogen is also responsible for post- harvest rot of the taro corms and fortunately *P. colocasiae* does not have a wide host range. *Xanthosoma* is found to be immune to leaf blight; but *Amorphophallus* has been reported with *P. colocasiae* infection (Misra *et al.*, 2008). This disease is more prominent in northern and eastern parts of India, which are potential taro producing areas. The disease appears occasionally in South India, but in serious proportions as per the reports of Misra and Chowdhury (1997).

2.3.1 Symptoms

As the name proposes, the most reliable and observed symptom is the appearance of blight on the lamina of taro leaf, but *P. colocasiae* also causes a postharvest corm rot. In several susceptible varieties, a petiole rot is also reported. The first and foremost symptom of taro leaf blight is the appearance of little dark or light brown spots on the adaxial leaf side of taro. These early lesions were often seen at the extreme leaf tips and they quickly enlarge, become zonate, circular and purplish-brown in colour. On the abaxial side, spots have a dry grey or water soaked appearance (Zhang *et al.*, 1994). The lesion margins were marked by a white powdery sporangia and orange or reddish brown exudates as droplets (Bandyopadhyay *et al.*, 2011). As the lesions increases in size, they coalesce and

25

rapidly destroy the leaves within 3-5 days after its initial symptoms, based on the weather conditions.

2.3.2 History of taro leaf blight

Leaf blight has become a major limiting factor for taro production in the Solomon Islands, Hawaii, Fiji, Western Samoa and in India causing up to 50 percent yield loss (Misra, 1999; Misra *et al.*, 2008). The geographic distribution of this disease is probably constrained to South East Asia and Pacific areas (Holliday, 1980). In India, leaf blight is reported to be a serious disease in many areas such as Kangra valley of Himachal Pradesh (Luthra, 1938), Assam (Chowdhury, 1944), Himachal Pradesh (Paharia and Mathur, 1961) and other states (Misra, 1999).

There exist only partial datas on the origin of *P. colocasiae* and the extent of taro cultivated areas (Ann *et al.*, 1986; Zhang *et al.*, 1994). It is suspected that Southeast Asia may be the *P. colocasiae* centre of origin as this is the world's centre of origin for many cultivated taro varieties and wild species too (Trujillo, 1967; Ko, 1979). The existence of *Phytophthora* A1/A2 mating type ratio of 1:1 can be a strong evidence for assuming its centre of origin (Zentmyer, 1988). However, it is believed that in India, only A1 mating type has been found (Narula and Mehrotra, 1980) while recently both mating types were observed in India (Misra *et al.*, 2011; Nath *et al.*, 2014). The presence of both the A1/A2 mating types of the heterothallic oomycete pathogen in the same taro fields not only elevates the chances of oospore formation and genetic recombination of *P. colocasiae,* but also may answer the mysteries regarding its origin.

2.3.3 Crop loss

Taro leaf blight is believed to have contributed to the considerable reduction in taro production and its dislocation in many regions of South East Asia. There were approximately 350 different taro varieties in Hawaii, prior to the arrival of leaf blight. The reports states that today the number of Hawaiian taros is less than 40 as a few have survived the disease (Trujillo, 1967).

The disease has severely affected taro production in Samoa (Gurr, 1993) and within the first year of disease introduction it had caused more than 95%

reduction in the taro supply to the local market. The disease quickly spread throughout the country harshly affecting all the available local varieties, but was harsh on taro variety *Niue*, which is having higher quality and taste.

This disease is reported to have eliminated taro cultivation in Papua New Guinea, both in the islands of Manus and Bougainville (Packard, 1975). Reductions in corm yield of 25-50% have been reported in various Pacific locations. There occurred 25- 50% yield reductions in Solomon Islands (Gollifer and Brown, 1974) and 25-35% of corm yield have been recorded in the Philippines regions (Brooks, 2008).

In India, the disease has become a limiting factor for production in all taro growing areas causing 25% to 50% yield loss every year (Jackson and Gollifer, 1980; Misra, 1997; Gadre and Joshi, 2003) which infect leaves, petioles, corms and cormels leading to heavy loss in yield which may exceed 60% in severe cases (Sahu *et al.*, 1989; Gurung, 2001).

2.4 PLANT PATHOGENIC OOMYCETES

Oomycetes are the most devastating plant pathogens. In appearance and behavior, they resemble fungi but they have independently evolved during evolution. While the fungi belong to the super group unikonts, in the tree of life, oomycetes belong to the sub group chromalveolates, of the straminopile lineage (Keeling *et al.*, 2005).

The *Phytophthora* genus consists of more than 100 described species, certain species have a narrow host range while others have a broad host range (Erwin and Ribeiro, 1996). The name *Phytophthora* is taken from the Greek words *Phyto* with meaning plant and *phthora* means destroyer. The genus was first described by Heinrich Anton de Bary in 1875 (Brasier, 2009). The genus *Phytophthora* is closely related to genus *Pythium* and both genera are water molds and classified in the family *Pythiaceae*, so named since the genus *Pythium* was described first. Oomycetes are responsible for economically devastating epidemics like Irish potato famine caused by *Phytophthora infestans* of 19th century which results in the death of 1 million Irish people (Gregory, 1983). Moreover,

economically vital diseases like root and stem rot by *Phytophthora sojae* (Sugimoto *et al.*, 2006), which causes decline in soybean production over several continents, black pod of coco caused by *Phytophthora palmivora* (Butl.) and *Phytophthora megakarya* Bras. Griff, became a serious limitation to coco production, and sudden oak death caused by *Phytophthora ramorum*, is destructing oak trees along the United States Pacific cost (Rizzo *et al.*, 2001).

The important characteristics feature of *Phytophthora* species are its hyphal swellings, biflagellate zoospores, sporangia, chlamydospores. The main sex organs of *Phytophthora* include antheridia, oospores and oogonia. The thallus is called mycelium as in fungi and is composed of hyaline, branched and coenocytic filaments. The major part of life history is primarily diploid whereas the higher fungi are haploid. The cell walls of *Phytophthora* are composed of cellulose and β-glucans and not chitin, which is the common cell wall component of the fungi (Bartnicki-Garcia, 1968). Mycolaminarin, a β- 1,3- glucan, is the characteristic storage carbohydrate. The zoospores are biflagellate; one is whiplash and the other is tinsel flagellum (Hemmesd, 1983). *Phytophthora* species are unable to generate sterols, therefore, for its sporulation it requires an exogenous β- hydroxy sterols source.

2.5 Phytophthora colocasiae

Raciborski first described the causal organism of taro leaf blight as *Phytophthora colocasiae* in 1890 from Indonesia. The mycelium is coenocytic, hyaline and inter- or intracellular. Moreover, the haustoria are found to be long, slender and unbranched. The optimum temperature and pH for proper growth of the pathogen is 28^0C and 6.5 respectively. Sporangia are semi- papillate, ovoid to ellipsoid shaped structures which are found at the end of short, sparingly branched or unbranched sporangiophores. These sporangiophores are very unbranched, slender and extremely narrow at the tip with about 50 μm in length. Depending on the weather conditions they germinate directly or indirectly. During indirect germination, biflagellate zoospores are released, which get converted to cysts and later germinates after 30 min (Trujillo, 1965; Misra, 1996). *P. colocasiae* had

become a devastating pathogen due to the abundant production of zoosporangia, cysts and zoospores.

The presence of both A1 and A2 mating types is necessary for the sexual reproduction of this heterothallic species. One mating type stimulate the production of sex organs like antheridia and oogonia in the opposite mating type by means of certain hormones produced by them. The base of oogonium is found to be attached by each antheridium, which gives a stalk like attachment (amphigynous). Haploid nucleus from the oogonium unites with a haploid nucleus in the antheridium, forming 18- 30 μm diameter diploid oospore.

P. colocasiae produces proteolytic enzymes like polygalacturonase, pectin methyl transeliminase and poly methyl galacturonase and theswe enzymes may play a major pathogenesis role in the *C. esculenta* (Aggarwal and Mehrotra, 1987). *Amorphophallus paeonnifolius* and black pepper were also reported to be the host for *P. colocasiae* (Paharia and Mathur, 1961).

2.5.1 Biology and Ecology

While comparing with other *Phytophthora* species, only very little work has been done on the biology and ecology of *P. colocasiae*. The disease spread is by sporangia and zoospores that are carried by rain or wind splash between plantings and plants. Most of the infections were found to occur between midnight and dawn (Putter, 1976). Daytime infections are rare and only possible during endlessly wet weather conditions. Germ tubes developing from either encysted zoospores or sporangia enter via stomata or directly penetrate the epidermis, during infection time. Through the mesophyll cells the oomycete spreads intercellularly, after its successful penetration. Usually within the 24 hours first symptoms appear and it is found that when the temperatures are within the range of 25–30 °C, under rainy or cloudy conditions the rate of symptom development is at its maximum. Symptom development is ceased or suppressed at an atmospheric temperature of 35 °C. The sporangia of *P. colocasiae* can move through the water, so wetland taro fields are severely affected by the pathogen. During dry periods The pathogen can survive in soil during its unfavourable situations like extremely dry weather conditions as chlamydospores or encysted zoospores (Quitugua and Trujillo, 1967). The fungal

mycelium will survive less than five days due to its short lifespan in soil. However, most sporangia in living host seldom survive more than a few days though some survive up to 2 weeks but the encysted zoospores of *P. colocasiae* can endure for several months in the absence of a vegetative material (Fullerton and Tyson, 2004).

2.5.2 Epidemiology

Leaf blight is observed in severe form in areas having high relative humidity and frequent rain fall, whereas warmer areas having little rain fall and relative humidity are comparatively free from this disease. Trujillo (1965) found that blight epidemics occurs maximum when relative humidity is 100% at night, 65% during the day time accompanied by a cloudy weather conditions and when night and day temperatures ranged between 20 - 22^0 C and 25 - 28^0C, respectively. Under such conditions taro leaves could be damaged by blight disease within 5-7 days.

2.5.3 Heterothallism and genetic variability

P. colocasiae causing taro leaf blight is a diploid heterothallic oomycete. It requires both its opposite mating types A1 and A2 for the formation of its oospores (Tyson and Fullerton, 2007). Heterothallic species of *Phytophthora* readily produce oospores in pairing of opposite mating types (Ko, 1979) and they evolve rapidly depending on the frequency of mating types by the recombine of different strains. There is no evidence for occurrence of oospore formation regularly in nature, however this event is common in culture.

ITS characterization and sequence analysis of *P. colocasiae* populations from fine spatial scales exhibited higher levels of DNA polymorphism at the ITS I regions. This result depicts that these pathogens are evolving continuously in the nature (Nath *et al.*, 2013). Mechanisms such as chromosome deletions, translocations and duplications are very common events in *Phytophthora* species (Goodwin, 1997) and this may be the case with *P. colocasiae* also.

Drenth *et al.* (1996) have discovered already the rapid mutation events in *P. sojae* and Goodwin (1997) identified several clonal lineages of *P. infestans*. is known to rapidly Asexual reproduction and formation of large number of

sporangia are common events in *P. colocasiae* under favorable conditions they germinate either directly or indirectly to produce motile zoospores. There are high chances for outsourcing events when the sporangia get dispersed to neighboring hosts by means of rain or wind splashes. There are two chances for outsourcing events. One is when two strains are mixed in culture (Drenth *et al.* 1995, 1996) and the other is when the two strains infect the same plant (Fry and Goodwin 1995). The higher levels of diversity exhibited by the pathogen are most likely due to recombination, random mutation, cropping patterns, exposure of the pathogen in diverse climates, hosts and pathogen movement within the country.

2.6 MOLECULAR MARKERS FOR GENETIC DIVERSITY STUDY

Molecular marker technologies have been employed extensively in many fields of plant pathology. They offer the possibility of fast, accurate identification and early detection of plant pathogen (Bridge *et al.*, 2003) and can answer many complex questions, such as the changes in their population structure and the population dynamics of the disease they cause (Hernandez-Delgado, 2009). Diversity among organisms is a result of mutations resulting from substitution of single nucleotides, insertion or deletion of DNA fragments, duplication or inversion of DNA fragments and can be due to recombination. According to Wang *et al.* (2009) any variation in nucleotides, genes, chromosomes or whole genomes of organism can be considered as genetic diversity. It can be assessed among different individuals within the same species (intraspecific), among species (interspecific) and between genus and families (Mittal and Dubey, 2009). Molecular markers act as powerful tools for the analysis of genetic diversity, based on DNA sequence polymorphisms. DNA polymorphisms evaluation techniques directly measure the genetic diversity of the particular organism. Since Mendelian inheritance is exhibited by molecular markers, it is possible to trace the evolutionary history of the species by phylogenetic analysis, studies of population genetic structures, genetic relationship and genome mapping studies. There are three classes of molecular markers: (a) nucleic acid hybridization based on complementary bases, (b) polymerase chain reaction (PCR) based on DNA amplification and (c) single

nucleotide polymorphisms (SNPs). The use of first technique, has been declined due to the difficulties involved in manipulating high throughput sampling and the high costs related to large scale genotyping in the case of SNPs. For the diversity studies, the cost effective PCR based techniques have been largely used.

2.7 MICROSATELLITES

SSR markers are stretches of DNA consisting of tandemly repeating motifs of variable lengths that are distributed in both noncoding and coding regions of the eukaryotic nuclear genome (Jarne and Lagoda, 1996; Powell *et al.*, 1996). PCR primer pairs were designed using the conserved regions flanking the repeats and the intervening repeat loci were amplified. Litt and Luty first call them as microsatellites and later as SSR by Jacob *et al.* (1991). Almost thirty years ago Microsatellites were detected in the genome of eukaryotes and they were considered as the most promising PCR-based molecular markers. Microsatellites appear as tandemly repeated motifs in the eukaryotic and prokaryotic mitochondria (Soranzo *et al.*, 1999) organellar genomes and chloroplast (Powell *et al.*, 1996) They are potentially the most informative molecular marker due to the high mutation rates and the main advantage of SSR markers are its easy and low-cost detection by PCR. Moreover, the laborious approaches and high cost to isolate microsatellite loci has been overwhelmed by new sequencing technologies. Many published loci could be transferred from related species and the chances of cross amplification across different species of the same genus makes SSR markers desirable. Using bioinformatics tools, large databases of EST and genomic sequences, could be screened and can be used for identifying SSR motifs. The co-dominant feature of SSR markers allow the researchers to differentiate between homozygotes and heterozygotes, unlike dominant markers like RAPD and AFLP that detect only the locus presence or absence.

SSR act as an excellent marker. Due to its hypervariability among related organisms. It is widely used for several applications, including genetic mapping, genotype identification, molecular tagging of genes analysis of genetic diversity, Marker Assisted Selection (MAS) and phenotype mapping (Tautz, 1989). There are

lot of evidence indicating that SSRs in non- coding regions are also functional (Mortimes *et al.*, 2005) and believed to be involved in regulation, gene expression and functional (Gupta *et al.*, 1994; Kashi *et al.*, 1997). This marker has been employed in several fields like germplasm conservation, plant and animal breeding programs, phylogenetic analyses, constructing linkage maps, identifying genes responsible for desired traits and mapping quantitative traits which are economically important.

Based on the number of nucleotide bases several authors had grouped SSR markers, like microsatellites which are short repeats with 10- 30 bases and minisatellites which are longer repeats with bases between 10-100. Further microsatellites have been grouped based on the type of repeat sequence like perfect, imperfect repeats and composite repeats. In perfect repeats they show only perfect repetitions, in imperfect repeats the repeated sequence is interrupted by different nucleotides that are not repeated and in composite repeats there are two or more different motifs in tandem. The composite repeats can be either perfect or imperfect. The most common choices for molecular genetic studies are found to be the sequences of di-, tri- and tetranucleotide repeats (Selkoe and Toonen, 2006).

Using polymerase chain reaction (PCR) in stringent conditions amplification of single loci can be performed, for the facilitation of data integration (Bravo *et al.*, 2006). Furthermore, microsatellites are transferable between species, highly polymorphic and distributed widely throughout the genome (Chistiakov *et al.*, 2006).

2.7.1 Isolation and analysis of SSR loci

Several protocols were recently published on the improved isolation methods of SSR loci (Zane *et al.*, 2002; Schena *et al.*, 2008). These microsatellite isolation protocols can be grouped into three following types like (i) the standard method of library construction and screening for the presence of repeated sequences; (ii) the SSR sequences are searched in a large set of sequence databases which is the so called automated method, where and (iii) High-throughput technologies based whole genome or partial genome sequencing.

Using the standard methods of SSR isolation the number of positive clones generated ranges from 0.04 to 12%, with birds having its lowest yield (Zane *et al.*, 2002). Even though, this method is highly efficient; the higher cost of microsatellite marker development makes it undesirable as it requires the evaluation of a huge number of clones to find those with repeated sequences from the total genomic DNA library. Ito *et al.* (1992) used biotinylated oligonucleotide for the screening of the plasmids of a restriction fragment library. The positive clones could be identified using streptavidin coated magnetic beads as plasmid and the oligonucleotide interact to form a triple helix. Later purification of the microsatellite-enriched plasmids was performed and transformation into *E. coli* were done. Sequence motifs that are capable of triple helix formation were only used for this technique.

The extension of the library of single-stranded genomic DNA using repeat specific primers can be used as a technique to increase the number of positive clones. Amplification of genomic libraries using primers such as biotinylated oligonucleotides, which were complementary to the microsatellite sequence were done by Paetkau (1999). The single-stranded biotinylated sequences were recovered with streptavidin bound to magnetic particles, made double-stranded and transformed into *E. coli*. In this case, there exist 100% enrichment efficiency for the dinucleotide $(CA)_{18}$.

Enrichment of microsatellite libraries using another methods as described by Yue *et al.* (2009) was found to be efficient. To normalize a pool of cDNA prior to cloning they used a duplex-specific nuclease and generated 30 times more positive clones than direct sequencing methods. Recently, Santana *et al.* (2009) and Malausa *et al.* (2011) demonstrated pyrosequencing to augment DNA libraries of several species and this methodology was found to be more effective, profitable and rapid than others.

The increased use of public DNA databases to search for repeated sequences has made microsatellite development and identification easier. Unspecific alignment tools, such as BLASTN (Altschul *et al.*, 1990) were used earlier for database searches. Later, the development of certain computer-based software

programs makes the SSR search easier (Mittal and Dubey 2009). The costs associated with microsatellite marker development get reduced due to this automated method, but is limited to species having available sequences in the databases.

The whole or expressed genome sequencing were done using the new high-throughput sequencing technologies (Abdelkrim *et al.*, 2009; Mikheyev *et al.*, 2010). These technology produces huge amount of sequences quickly and it does not require creation of libraries.

2.7.2 Transferability of SSR markers

Since the flanking regions are highly conserved across taxa, microsatellites are transferable and allow cross-species amplification. So the primers synthesized for one species can be used for different species of the same family or genus (Rico *et al.*, 1996; Peakall *et al.*, 1998). The transferability genomic DNA libraries derived SSRs were found to be lower than EST databases derived SSRs. As the EST-SSRs developed from the expressed gene sequences, they are highly conserved over a number of species than the non-coding regions (Varshney *et al.*, 2005).

2.7.3 Limitations of SSR markers

If any mutations or locus deletion occur in the annealing primer site, that can result in prevention of heterozygous identification or locus amplification and lead to incorrect segregation rates and allele frequencies estimations. Deviations from the random association of alleles in a population, is considered as another important limitation of SSR markers which are primarily due to high levels of inbreeding and population substructuring (Goss *et al.*, 2014). It is highly problematic for paternal exclusion and population studies

2.8 SSR DIVERSITY IN *Phytophthora*

Forty SSR primer pairs were designed by Zhu *et al.* (2004) and tested on 5 different *P. sojae* strains. Upon PCR amplifications, 33 primer pairs produced distinct bands and 28 out of 33 functional primer pairs were able to produce

characteristic SSR bands with expected size. Among them 15 primer pairs (45.5%) when tested on the 5 *P. sojae* strains, had produced significant level of polymorphisms.

Studies by Lees *et al.* (2006) has examined 90 *P. infestans* isolates and they exhibited significant SSR diversity with 2–9 alleles per locus having an average of 3·9. 68 novel genotypes were identified in that study. All loci were successfully amplified when genotyped with other species of *Phytophthora*. Majority of the loci were found to be polymorphic, which depicted their transferability for the study of other related taxa potentially. Studies by Chun- Fang *et al.* (2016) estimated the genetic variation of *P. infestans* isolates using 8 SSR markers. The seven *P. infestans* populations exhibited an average SSR diversity of 0.39 to 0.49.

Primers designed by Schena *et al.* (2008) were tested for representing the diversity across the genus and the possibility for cross species amplification. Microsatellite primers have been used to study the reproductive biology and genetic structure of oomycetes species including *P. cinnamomi*, *P. infestans*, and *P. ramorum.* This can amplify *P. sojae* with 33, *P. ramorum* with 17 and *P. infestans* with 8 SSRs in their target region. Overall, these primers were able to sequence 171 target regions having 211 SSRs with a repeat number of 3 to 16. The most common repeat motifs were (AGC)n, (AGG)n and (AAG)n. This data indicates that cross-specific amplification of SSR loci can be utilized for studying the diversity exhibited by other *Phytophthora* species.

The diversity analysis done by Biasi *et al.* (2015) identified and screened 5118 SSR loci in the 14 *P. nicotianae* isolate's genomic assemblies. The primers were synthesized for the amplification of 17 SSR markers distributed among the different contigs. Genetically distant pathogen isolates were amplified as these loci were highly polymorphic. With two alleles a higher degree of heterozygosity was established in 67% of the primer combinations. It depicts variation in its ploidy by detecting three separate alleles for a single locus.

Vettraino *et al.* (2017) studied the diversity of eighty-eight *P. lateralis* isolates, representing 4 populations with five selected SSR primer combinations, generating sixteen 'alleles' overall among five loci.34 potential SSR markers were

screened in this study. From this, only five (14.7 %) primers were considered for the diversity analysis and genotypic variation study among the different populations. The minimum spanning network and *Fst* values in this study reflected the divergence exhibited by the 4 populations.

Biasi *et al.* (2016) studied the genetic diversity exhibited by 231 *P. nicotianae* isolates belonging to 14 populations from different hosts, using microsatellite markers. By exposing a strong relation between host of recovery and genetic grouping, a total of 99 multilocus genotypes (MLG) were identified in this study. An interesting fact is that most of the MLG were found to be associated strongly with a single host genus. Moreover, a significant difference in the population structures were also revealed. Irrespective of their geographic origin, isolates collected from *Citrus* were found to be genetically identical and were extensively studied by its inbreeding coefficients and high genetic uniformity.

Brurberg *et al.* (2011) analyzed 200 *P. infestans* isolates from different fields of Nordic countries using 9 SSR markers. Among the 9 SSR loci, 49 different alleles were detected in their study. Based on 7 loci, 169 multilocus genotypes were identified among the 191 *P. infestans* isolates. For the four Nordic countries isolates, the diversity indices, quantified by Shannon's diversity index (H_s), were found to be 0.95. The majority of variation was found within the 4 Nordic countries as the F_{ST} value was very low (0.04).

Knapova and Gisi, (2002) studied the phenotypic and genotypic structure of *P. infestans* populations on potato and tomato in France and Switzerland. Isolates of *P. infestans* from Switzerland and France were included in this study. A total of 134 *P. infestans* isolates were collected from potato and 42 from tomato fields. Morphological and phenotypic characteristics such as phenylamide fungicides sensitivity, mating type identification, virulence assay on potato, and pathogenic fitness were identified in the present study. Assessment of genotypes was performed with RFLP, AFLP and SSR marker based studies. SSR and AFLP markers based variability study revealed the chances of recent sexual recombination, selection and migration events in the field populations.

Montarry *et al.* (2010) revealed the population structure of 220 French *P. infestans* isolates collected using 8 SSR markers. Upon clustering analysis, French *P. infestans* populations were differentiated into two clusters of isolates. The higher F_{ST} value ($F_{ST} = 0.19$) suggests the possibility of *P. infestans* introductions into France.

Wu *et al.* (2012) studied the molecular characterization and diversity analysis of *Phytophthora infestans* populations collected from the North China region using 15 SSR markers. In this study an average of 3.8 alleles were found per locus. Diversity analysis on 134 strains of *P. infestans* from 4 populations, revealed a low genetic diversity. The Shannon's diversity index and average heterozygosity was found to be 0.26 and 0.162 respectively. The lower values of average heterozygosity and Shannon's diversity index indicates the possibility of clonal pathogen populations in North China.

Stroud *et al.* (2015) studied the genetic diversity of *P. infestans* populations collected from potato and tomato fields in the British gardens. They demonstrated higher genetic diversity but here exists no indication of host specialization. They studied the genetic diversity of British *P. infestans* populations using 12 multiplexed SSR markers. Between the garden tomato and potato hosted samples, there exists only 2% of molecular variance. Moreover, they had revealed a high degree of genetic variation in *P. infestans* populations from British gardens.

In the Swedish experimental fields, Widmark *et al.* (2011) studied the late blight epidemic dynamics and sexual reproduction events in *P. infestans* populations. Six *P. infestans* isolates collected from different potato farm were sexual reproduction of the pathogen was suspected. These isolates were later used for inoculating healthy Swedish potato fields. Characterization of resulting 151 isolates were performed using SSR markers. The genotypes act as inoculums constituted more than 80% of the resulting genotypes and 3 different genotypes marked the remainder. Six novel genotypes were identified in the following year from the same field.

Wang *et al.* (2013) studied EST-SSR based diversity of *Phytophthora infestans* collected from Gansu province in China. They had designed and synthesized eleven pairs of EST- SSR primers and analyzed 63 isolates of *P. infestans* using polymerase chain reaction. Nine pairs of primers (81.8%) amplified and produced polymorphic SSR bands. Moreover, the 63 isolates of *P. infestans* were divided into 20 genotypes by cluster analysis.

Zhu *et al.* (2004) identified 415 SSRs in 369 ESTs of *P. sojae* with an average density of one SSR per 8.9 kb of screened EST sequence. Thirty-three primer pairs produced successful amplifications upon PCR when 40 primer pairs tested on 5 *P. sojae* strains. These 5 *P. sojae* strains upon cluster analysis, were grouped in to 3 clusters. Among the 20 EST SSR primers, 15 primer pairs (45.5%) detected polymorphism upon PCR amplifications.

Chimelarz *et al.* (2014) studied the genetic diversity of 96 *P. infestans* isolates from Polond using 12 SSR markers. SSR marker based genotyping revealed extensive genetic diversity within the *P. infestans* populations of Polond. Among the 96 *P. infestans* isolates, 66 genotypes were found to be unique and 49 of them are only seen in single isolates.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled, "Genetic diversity analysis of *Phytophthora colocasiae* using SSR markers" was conducted at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during October 2016 - August 2017. Details regarding to the experimental materials and methodology used in the study are elaborated in this chapter.

3.2 SAMPLE COLLECTION

The leaf samples with typical leaf blight symptom of water soaked lesions (Plate 1) were collected on a random basis from taro (*Colocasia esculenta* L. Schott.) growing fields at ICAR - Central Tuber Crops Research Institute (CTCRI), Sreekariyam, Thiruvananthapuram. Young taro leaves with multiple leaf blight lesions were used for the pathogen isolation at fine spatial scale. Leaves from "Sree kiran" variety with no observable symptoms were also sampled and used to carry out the pathogenicity test. *P. colocasiae* isolates available at the repository were revived and sub-cultured in carrot agar medium. A total of 37 *P. colocasiae* isolates collected from different geographical locations of India and being maintained at ICAR-CTCRI microbial repository along with the 15 cultures isolated from ICAR-CTCRI farm (Table 1) were used in this study

Table 1. Isolates of *Phytophthora colocasiae*

Sl. No.	Isolate code	location	District/ sampling site	Year of collection	Colony morphology on PDA
1	CTCRIPC1	Kerala	Kottyam	2011	Plain
2	CTCRIPC13	Kerala	Thrissur	2016	Plain
3	CTCRIPC31	Kerala	Idukki	2010	Flat with concentric rings
4	CTCRIPC14	Kerala	Pathanamthitta	2011	Plain
5	CTCRIPC34	Kerala	Paravur, Kollam	2016	Irregular pattern
6	CTCRIPC24	Kerala	CTCRI, Block2	2016	Plain with irregular concentric rings

7	CTCRIPC22	Kerala	Ernakulam	2016	Plain
8	CTCRIPC2	Kerala	Paravur, Kollam	2016	Irregular pattern
9	CTCRIPC21	Kerala	Karavur, Kollam	2007	Irregular pattern
10	CTCRIPC7	Kerala	Chelavur, Calicut	2016	Plain
11	CTCRIPC5	Kerala	Ayoor, Kollam	2011	Irregular pattern
12	CTCRIPC20	Kerala	CTCRI, Block1	2016	Uniform without pattern
13	CTCRIPC26	Kerala	CTCRI, Block1	2016	Uniform without pattern
14	CTCRIPC27	Kerala	CTCRI, Block1	2016	Uniform without pattern
15	CTCRIPC28	Kerala	CTCRI, Block1	2016	Uniform without pattern
16	CTCRIPC29	Kerala	CTCRI, Block1	2016	Uniform without pattern
17	CTCRIPC30	Kerala	CTCRI, Block1	2016	Uniform without pattern
18	CTCRIPC31	Kerala	CTCRI, Block1	2016	Uniform without pattern
19	CTCRIPC32	Kerala	CTCRI, Block1	2016	Uniform without pattern
20	CTCRIPC44	Kerala	CTCRI, Block1	2016	Uniform without pattern
21	CTCRIPC47	Kerala	CTCRI, Block1	2016	Uniform without pattern
22	CTCRIPC37	Odisha	Khandapara	2007	Cottony
23	CTCRIPC19	Odisha	Nayagarh	2007	Cottony
24	CTCRIPC23	Odisha	Anandpur	2007	Cottony
25	CTCRIPC8	Odisha	Puri	2007	Cottony
26	CTCRIPC9	Odisha	Puri	2007	Cottony
27	CTCRIPC6	Odisha	Salepur	2008	Cottony
28	CTCRIPC11	Odisha	RC, CTCRI	2009	Cottony with concentric rings
29	CTCRIPC18	Odisha	RC, CTCRI	2009	Cottony with concentric rings
30	CTCRIPC35	Odisha	Khandapara	2007	Cottony
31	CTCRIPC45	Odisha	Nayagarh	2007	Cottony

32	CTCRIPC3	Assam	Nellie Road	2007	Cottony with concentric rings
33	CTCRIPC46	Assam	Nellie Road	2007	Cottony with concentric rings
34	CTCRIPC38	Assam	Nellie Road	2007	Cottony with concentric rings
35	CTCRIPC43	Assam	Nellie Road	2010	Cottony with concentric rings
36	CTCRIPC45	Assam	Nellie Road	2010	Cottony with concentric rings
37	CTCRIPC41	Assam	Nellie Road	2010	Cottony with concentric rings
38	CTCRIPC17	Assam	Sikkim	2011	Cottony with concentric rings
39	CTCRIPC15	Assam	Sikkim	2011	Cottony with concentric rings
40	CTCRIPC40	Andhra Pradesh	Veerwada	2010	Plain with irregular concentric rings
41	CTCRIPC42	Andhra Pradesh	Veerwada	2010	Plain with irregular concentric rings
42	CTCRIPC39	Andhra Pradesh	East Godawari	2011	Plain with irregular concentric rings
43	CTCRIPC33	Andhra Pradesh	East Godawari	2011	Plain with irregular concentric rings
44	CTCRIPC4	Andhra Pradesh	Parudin pallam	2010	Plain with irregular concentric rings
45	CTCRIPC25	Andhra Pradesh	Parudin pallam	2010	Plain with irregular concentric rings
46	CTCRIPC10	Andhra Pradesh	Veerwada	2011	Plain with irregular concentric rings
47	CTCRIPC12	Andhra Pradesh	Veerwada	2011	Plain with irregular concentric rings

3.3 ISOLATION OF PATHOGEN

Taro leaves showing the typical leaf blight symptom of water soaked lesions were collected and washed with sterile water for removing the surface impurities. Small fragments (1-2 cm) of diseased tissue along with some healthy tissue were excised with a sterile scalpel and surface sterilized with 70% ethanol for 1 minutes, later with 1% sodium hypochlorite solution for 2 minutes. The surface sterilized leaf bits were rinsed twice with sterile distilled water and blotted dry using sterile Whatman filter paper in a laminar flow hood. These leaf tissues were then transferred aseptically into sterilized petri dishes containing solidified potato dextrose agar (PDA) and incubated at 28 ^{0}C in incubator for mycelial growth. After three days of incubation mycelial growth was observed along with diseased leaf bits. Hyphal tips from the mycelia were transferred using autoclaved pipette tips to the water agar medium and finally to carrot agar slants. Based on the sporangial and mycelial characters, the isolates were identified initially as *Phytophthora colocasiae* through standard mycological keys (Waterhouse 1963; Hemmes, 1993) and by comparing the isolates with reference isolates maintained at ICAR- CTCRI, Thiruvananthapuram, India

3.3.1 **Revival and subculture of *P. colocasiae* isolates**

P. colocasiae isolates collected from various Indian states were conserved in the ICAR-CTCRI repository, from which 32 isolates were selected for the present study. These isolates were revived by inoculating the cultures artificially on leaf blight susceptible variety 'Sree Kiran'. After 2-3 days the symptom appeared on the inoculated leaves. Pathogens were re-isolated from the infectious leaf tissue as described above in ampicillin (100 mgl^{-1}) supplemented PDA medium. *P. colocasiae* isolates were then sub-cultured in carrot agar (CA) medium.

3.4 CULTURE CHARACTERIZATION

Growth characteristics, sporangial production and colony morphology were studied on five different culture media: Carrot agar, Potato dextrose agar,

Czapek dox agar, Oat meal agar (OMA) and V8 juice agar. A 5 mm disc of actively growing mycelia, from water agar culture were transferred to the centre of petri dishes containing above mentioned media. Plates were incubated in dark at 28^0C for one week. After incubation, based on mycelial texture and colony morphology studies, *P. colocasiae* isolates were characterized on different culture media. For each isolate, 3 replicates have been used at similar incubation conditions to confirm the culture characteristics. Isolates were grown at different temperature conditions like 15, 20, 25, 28, 35, 40^0C in triplicates to study the effect of temperature on growth of the pathogen.

3.4.1 Sporangial morphology

For the examination of sporangial morphology, spores were generated by growing the isolates on carrot agar plates at 27 °C for 5 days. Sporangial induction was done by culturing 1 cm mycelial disc on 15-20 ml sterile distilled water under light for 3 days (Aragaki *et al.*, 1967). The spores generated were stained with lactophenol cotton blue and observed under a light microscope (Nikon Eclipse 80i Nikon Corporation, Tokyo, Japan). The dimensions (length and breadth) of sporangia were measured, at 40 X magnification for each isolates.

3.5 VIRULENCE ASSAY

To confirm the virulence of the pathogen, a floating leaf disc method of pathogenicity test using leaf blight susceptible variety 'Sree Kiran' was performed. Five leaf discs (5x5 cm) were floated in glass petri plates containing sterile distilled water. Mycelial disc cut from the *P. colocasiae* cultures were inoculated on each floating leaf disc. As control treatments leaf bits containing sterile agar plugs were used. The inoculated leaf discs were kept for incubation at 28°C for 4 days and observed continuously for symptom appearance. The assay was repeated twice and re-isolation was made from all resulting lesions according to Koch's postulate.

3.6 NUCLEIC ACID BASED DETECTION

PCR using species specific primers (Nath *et al.*, 2014) was performed on isolated genomic DNA of all the 47 isolates, to confirm the isolates at species level. Universal ITS1 and ITS4 primers (White *et al.* 1990) were used for the identification of the pathogen at genus level.

3.6.1 Genomic DNA isolation

For the genomic DNA isolation, *P. colocasiae* isolates were grown for collecting mycelia in potato dextrose broth medium at 28^0C for one week. Two to four actively growing mycelial discs were inoculated to 150 ml sterilized potato dextrose broth in an Erlenmeyer flask and the cultures were kept on a rotary shaker (100 rpm) for incubation. Following incubation, mycelia were collected by cheesecloth filtration and kept for drying in sterile Whatman filter papers. These dried mycelia were used immediately for genomic DNA isolation.

3.6.1.1 Genomic DNA purification kit (Thermo Fisher Scientific, Fermentas, EU)

Dried mycelium weighed 80-100 mg was taken and ground into fine power with liquid nitrogen using mortar and pestle. The powdered tissue was mixed with 400 µl of lysis buffer and transferred to a sterilized micro centrifuge tube. Then the samples were kept for 10 min incubation at 65°C in a waterbath. After adding 600 µl of chloroform, gently invert the tubes for 4-6 times and centrifuged the sample at 10,000 rpm for 2 min. The 10x concentrated precipitation solution (80 µl) was diluted with 720 µl of sterile deionized water. After centrifugation, DNA containing upper aqueous phase were collected on to a fresh micro centrifuge tube. In to this, freshly prepared 800 µl precipitation solution was added. The tubes were mixed gently mixed by several inversions for 2 min and finally centrifuged at 10,000 rpm for 2 min. After centrifugation, the supernatant was discarded completely. Finally, the DNA in the pellet form was dissolved with 100 µl of NaCl solution. 300 µl of ice cold ethanol was used to precipitate DNA. For the complete precipitation of DNA, the tubes were kept for

10 min incubation at -20°C. To pellet down DNA, the tubes were centrifuged at 10,000 rpm for 4 min. Inorder, to remove impurities, DNA pellet was washed twice with 70% ethanol. The quality of the isolated DNA dissolved in 100 µl sterile deionized water was checked on 1% agarose gel.

3.6.1.2 *Manual Methods of DNA Isolation*

3.6.1.2.1 Genomic DNA extraction method for Phytophthora (Cooke and Duncan, 1997)

Young mycelia of *Phytophthora* was harvested from PDB medium and dried using sterile Whatman filter paper. 100mg of mycelium was taken in a sterilized micro centrifuge tube with 50mg of sterile sand and 10 mg Polyvinylpyrolidone (PVP). These mixtures were ground finely using plastic Treff eppendorf homogenizers with 750µl extraction buffer. The homogenized mixture was kept for centrifugation at 13,000 rpm for 5 min. Following centrifugation, the supernatant was collected in a sterile micro centrifuge tube. 500µl of Tris saturated phenol: chloroform: isoamyl alcohol (25:24:1) mixture was added to it and inverted gently for 2 minutes. Centrifugation was carried out at 13,000 rpm for 5 minutes and the upper aqueous layer containing DNA was collected to sterile eppendrof tubes. After adding equal amount of isopropanol to the tubes, centrifugation was done at 12,000rpm for 10 minutes. After completing the centrifugation, the supernatant was discarded. The DNA pellet was washed twice in 70% ethanol, to remove impurities. Finally, the tubes were centrifuged at 10,000 rpm for 2 minutes and air dried the pellet. DNA was resuspended in 100µl sterile distilled water and after adding 3µl RNaseA (5mgml^{-1}) the tubes were kept for 30 min incubation at 37^{0}C. The quality of the isolated DNA was checked on 1% agarose gel.

3.6.1.2.2 *Modified Cooke and Duncan Genomic DNA isolation method*

Eighty to hundred mg fresh mycelium was ground to a fine powder using liquid nitrogen with a pre- sterilized mortar and pestle. One mL of pre- warmed extraction buffer, 20 µl β- mercaptoethanol and 0.25g PVP were added and mixed

with the samples. All the samples were transferred to 2 mL eppendorf tubes and centrifuged at 10,000 × g for 1 min, supernatant was collected in a fresh tube and pellet was discarded. 5µl of protenase K (10mg ml^{-1}) was added to each tube and vortex the samples thoroughly for 1 min. Place the tubes in a water bath for incubation at 65^0C for 20 min and then 90^0C for 10 min. Later the samples were kept for centrifugation at 10,000 × g for 15 min. After centrifugation, the supernatant was collected in a sterile tube and pellet was discarded. Five µl RNase A (10mgml^{-1}) was added to each tube and incubated at 37^0C for 20 min. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernatant and inverted the tubes for 20-30 times. The tubes were centrifuged for 15 min at 10,000 × g, upper aqueous layer was transferred to clean tubes without disturbing the middle layer. Equal volume of chloroform: isoamyl alcohol (24:1) was added to the tubes and samples were centrifuged at 10,000 × g for 15 min. In to the upper aqueous layer, 250 µl 3M sodium acetate and 750 µl ice cold isopropanol were added and inverted the tubes gently to precipitate DNA. Place the tubes in -20 ^{0}C for 20 min. To pellet the DNA, centrifuge the tubes at 10,000 × g for 15 min and discard the supernatant. The pellet was dissolved in 500 microlitre of 70% ethanol and centrifuged at 8,000 × g for 5 min and repeat this step twice to remove the impurities present in the pellet. The nucleic acid pellet was air- dried and dissolved in 30-50µl of nuclease free TE buffer according to the size of the pellet.

3.6.2Analysis of the extracted DNA

3.6.2.1 Agarose gel electrophoresis

The most familiar technique for assessing the integrity of total nucleic acid (DNA / RNA) preparation is to run a mixture of loading buffer with sample aliquot on an ethidium bromide stained agarose gel. Agarose of 0.8 - 1.5 percentage was prepared in 1X TAE buffer (Appendix III) for this purpose, then heated in microwave oven until completely melted and 0.5 µl per litre EtBr was added for the visualization of DNA/RNA. The solution at 60°C was poured on to a sample comb containing casting tray and kept for solidification at room temperature. Then the comb was removed followed by inserting gel into the

electrophoresis chamber and covered with buffer. An aliquot of the DNA/RNA sample (3 µl) mixed with the loading dye was loaded in each of the wells of the gel. The gel was run at 85 V (BIO RAD Power Pac HV, USA) for 40 min. The gel was then visualized under UV light and the image was documented using Alpha Imager (Alpha Innotech, USA).

3.6.2.2 Analysis using Nano drop spectrophotometer

The Thermo Scientific NanoDrop™ 1000 Spectrophotometer was used to measure the quality and quantity of samples with high accuracy and reproducibility. Subsequently, the concentration of the isolated DNA samples ng/µl, absorbance at 260nm, 280 nm and the ratio of absorbance at 260 nm to 280 nm were also measured and recorded for further calculations.

3.6.3PCR analysis with *Phytophthora colocasiae* species specific primers

The genomic DNA isolated from 47 samples were used for PCR amplification with species specific primers (Nath *et al.*, 2014). Primer concentration and reaction mix was optimized as listed below:

PCSP- R (forward primer) sequence: 5' CAGATGAAGAGGTCCTGTGAGG 3'

PCSP- R (reverse primer) sequence: 5' AGGGAGTTGGCACAACCATT 3'

The reaction mix was:

1X Taq buffer (With 1.5 mM $MgCl_2$)	:	2.5 µl
dNTP mix (100 µM)	:	0.5 µl
Forward Primer(20 ng)	:	0.5 µl
Reverse Primer(20 ng)	:	0.5 µl
Taq polymerase (1U)	:	0.5 µl
Template DNA (50 ng/ µl)	:	2.0 µl
Deionized Water	:	18.5 µl
Total Volume	:	25 µl

After the preparation of the reaction mix, it was vortexed (Labnet vortex mixer, USA) and flashed down. The PCR was carried out in Agilent Technologies sure Cycler 8800 (USA). The PCR programme was set with a 95 °C initial denaturation step for 2 min, then 30 cycles of 95 °C denaturation for 30 s, 55°C annealing for 45 s and elongation at 72 °C for one min. The final elongation was carried out at 72 °C for 8 min. Later on agarose gel (1.5%), along with 1 kb plus Gene Ruler DNA ladder (Fermentas), the PCR products were resolved and kept for visualization under UV and the image was documented with Gel DOC system (Alpha imager, Alpha Innotech, USA).

3.6.4 Genetic diversity analysis at fine spatial scale

Five *P. colocasiae* cultures were isolated from multiple leaf blight lesions of single taro leaf at fine spatial scale. These cultures were used for testing the diversity analysis of the pathogen at fine spatial scale by ITS characterization and sequencing.

3.6.4.1 PCR analysis with ITS primers

The genomic DNA isolated from five *P. colocasiae* isolates collected at fine spatial scale were used for amplification of the rDNA- ITS region using ITS1 (5' TCC GTA GGT GAA CCT TGC GG 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') primers (White *et al.*, 1990). A 25 µL reaction volumes for the ITS amplification, contains template DNA (50 ng), each deoxynucleotide triphosphate (100 µM), each primer (20 ng), $MgCl_2$ (1.5 mM), 2.5 µl of 1 x Taq buffer and 1 U of Taq DNA polymerase (Merck GeNei, Bangalore, India). The Taq buffer is composed of 50 mM KCl, 10 mM Tris HCl of pH 9.0 and 0.01% gelatin. PCR amplifications were completed in an Agilent sure cycler 8800 thermal cycler (Agilent Technologies, Columbia, MD, USA). The thermal profile for ITS amplification was designed as 95°C initial denaturation for 2 min, then 35 cycles of 95°C denaturation step for 30s, 54.1°C annealing reaction for 1 min, 72°C elongation for I min 30 s, and a final elongation of 72°C for 8 min. The ITS amplification products were resolved on a 0.5 µg ml^{-1} ethidium bromide stained

1.2% agarose gel and the gel image was caught through an alpha imager (Alpha Imager, Alpha Innotech, San Leandro, CA, USA).

3.6.5 Gel elution of PCR amplified fragments

Extraction of the PCR products was achieved with GeneJET gel extraction kit (Thermo Scientific, USA). The PCR products were resolved on agarose gel (1.2%) and the amplicon was excised from the gel using a clean sharp scalpel. The gel slice was placed into a pre- weighed 1.5 ml tube and its weight was recorded. After adding two times the volume of binding buffer, the gel slices were kept for incubation at 60 °C for 15 min. This incubation was continued in a water bath till the gel slice get dissolved completely. In order to facilitate the melting process, the contents of the tube were mixed by inversion every three min. After complete dissolution, 800 µl of the gel solution was added to the purification column and centrifuged at 14,000 rpm for one min. The column was placed back into the same collection tube, after discarding the flow through. After addition of 100 µl of binding buffer to the column, it was centrifuged at 14,000 rpm for one min. Again, the column was placed back into the collection tube, after discarding the flow-through. Later centrifugation was performed for 1 min, after adding 700 µl of the wash buffer in to the column. Again the flow-through was removed and the empty column was centrifuged for an additional one min to completely remove any residual wash buffer present in the column.

After the column was transferred into a new collection tube, it was air dried for 10 min. The purified DNA was eluted by adding 50 µl of the elution buffer to the purification column membrane followed by centrifugation for one min. After elution, the column was discarded and the purified DNA was stored at -20 °C. The gel elute was subjected to PCR using the same reaction mix under required cycling conditions. The products of PCR were assessed using agarose gel (1.2%).

3.6.6 DNA sequencing

Gel elute of PCR product were sequenced at the Eurofins genomics, Bangaluru, India. From the obtained nucleotide sequences, primer sequences were removed and low quality reads were corrected through screening process. Later

these sequences were transformed into consensus sequences and finally aligned with Geneious Pro software R10.0.7 (Kearse*et al.*, 2012). To confirm the identity of the isolates, these sequences were analyzed with NCBI BLASTn (Altschul*et al.*, 1990). Further, the sequence alignment was performed using CLUSTAL W software (Thompson *et al.*, 1994).

3.7 SSR ANALYSIS

Genetic diversity analysis of 47 *Phytophthora colocasiae* cultures collected from different geographical locations of India were analysed using reported 10 SSR markers (Schena *et al.*, 2008).

3.7.1 Primer screening

The following 25 SSR primer pairs were used for primer screening (Table 2). These primers were screened by performing gradient PCR by selecting temperatures between 48- 64^{0}C. Only 10 primer pairs producing clear, reproducible polymorphic bands were selected for further SSR analysis of *P. colocasiae* isolates.

Table 2. List of reported 25 SSR primers (Schena *et al.*, 2008) used for screening

Forward Primers	Reverse Primers	SSRs
S1F ACGACGTGTCCAAGAACCAC	S3R ATGTTGACCGTGTTCTGCTG	$(CCG)_7$;$(AGC)_4$; $(AGC)_{14}$
S4F AARATGACGTGGACKGAGAG	S5R TGATSGTGGAGAARCTCATCT	$(AAC)_{14}$
S6F GGAGTTCGCCATCAACAACT	S7R TCAGCTTCTGTCGRTCGAC	(AAG)14
S10F GCGSTACGAGACCTGGAC	S11R GACTCRCCCTTCGACTCSTC	(CAG)14
S12F GGAGGCCGAGTCGGARTA	S13R TAYTCCGACTCGGCCTCC	(AGC)14
S14F GACGCMSYYGAGTGGAAAG	S15R ATTTKGSACAGATACCGACG	(AAG)15
S16F TCTACGTGAATGCCATGAGG	S17R CGTTCAGCTTCTGTCGATCR	(AAG)15

S18F YACCATCTCCAACCTGCTG	S19R CACCACCTCGAGTAGCTCCC	(AGC)7; (AGG)13
S19F GGGAGCTACTCGAGGTGGTG	S20R TCGTCTCAATCTCKGACTGA	(AGC)6
S21F ATCTGGGCTTCCASGAGGT	S22R CTGATCCTCCGCCACAY	(AAG)12; (ATC)6; (ATC)4; (AAG)5
S23F GACTCGGACTCGGACGAC	S25R CTCCTGCTCKTCTTTCAGGC	(AGG)7; (AAG)10; (GAG)4; (AAG)12; (GAG)5; (AGA)6
S23F GACTCGGACTCGGACGAC	S37R CTTRCCBTCCTTGTCCTTYT	(AGG)7; (AAG)10; (GAG)4; (AAG)12; (GAG)5; (AGA)6
S27F GAAGCGCGGGCGWGT	S31R TCCTCCTCTTCTTCTTCGTCW	(AAG)4; (AGG)4; (ACG)4; (AGG)11
S29F MGCAAGAAGGCGTCGTA	S30R CCTTCATCATGAGCTTCTGG	(AGG)4 (AAG)11
R1F GYGGCGGTGGCTACAGYG	R3R CTGCTGYTGCTGGTTGAAAG	(ACC)4; (ACC)5; (ACC)4
R3F CTTTCAACCAGCARCAGCAG	R4R GTTCATCATGCCWCCCATR	(AGC)8
R4F YATGGGWGGCATGATGAAC	R5R AGGACCAGGAGATGGAGGAC	(AGC)4; (AGC)12; (AGC)4
R7F TGTTCCARACCCGCTTCC	R7F TGTTCCARACCCGCTTCC	(ACG)9; (AAC)5; (AGC)10
R10F GGAGATGACGGAAGATGACG	R11R CCATCGAARTACATSACACGA	(AAGCC)4; (AGG)9; (AAG)7
R15F CCGGAGCGCGTGGA	R16R GGTAGTTGAGCGGCTTCTTG	(CCG)6
R16F CAAGAAGCCGCTCAACTACC	R17R TAACGGATCAGCTCTTGCTG	(ATC)4; (AGG)8
I3F GCCTGTGGAYGAGAATGGYS	I4R CAGATCCACGACACCRGGY	(AAG)8
I5F CATCAACAAGTGCTCGTWCS	I6R TAGTCRAYGTTCTTGTTGTTCA	(AGC)5; (AGC)8
I7F GHGTGGGCGAGTACTCCAAG	I8R AAGCTGGCTATRWACACTGCCG	(AG)9
I9F GCATYGGGTCGTTCCTGTA	I10R AGHGTGCAGTACAGACCCGC	(AAG)11

3.7.2 Optimization of PCR conditions

The genomic DNA of *P. colocasiae* isolate (Isolate code CTCRIPC21) were taken initially for primer screening. Ten SSR Primer pairs which were able to produce reproducible polymorphic bands were selected for the study. The composition of the reaction mixture was as follows:

1X Taq buffer (With 1.5 mM $MgCl_2$)	:	2.5 µl
dNTP mix (100 µM)	:	0.5 µl
Forward Primer(20 ng)	:	0.5 µl
Reverse Primer(20 ng)	:	0.5 µl
Taq polymerase (1U)	:	0.5 µl
Template DNA (50 ng/ µl)	:	2.0 µl
Deionized Water	:	18.5 µl
Total Volume	:	25 µl

After the preparation of the reaction mix, it was vortexed (Labnet vortex mixer, USA) and flashed down. The PCR was carried out in Agilent Technologies sure Cycler 8800 (USA).

3.7.3 Gradient PCR for standardizing annealing temperature

The selected primers were taken one by one for screening to determine the annealing temperature for each primer pairs. Gradient PCR was performed for selected primer pairs in which the temperature ranged between 48°C to 64°C. The amplicons were further subjected to agarose gel electrophoresis to determine the annealing temperature.

3.7.4 PCR using SSR primers

After primer screening, 10 SSR primers, which gave clear and polymorphic bands were chosen for analysis. The PCR programme was set with a 95 °C initial denaturation step for 2 min, then continuous 30 cycles of 95 °C denaturation step for 30 s, annealing temperature which was found to be optimum for each primers for 45 s and a 72 °C elongation for one min. The final elongation step was carried out at 72 °C for 8 min. Later, the PCR products were allowed to run in an agarose gel of 2% concentration with 1 kb plus DNA ladder (Fermentas) using a voltage of 100 V for about 2 hours. The PCR products were visualized under UV light and the image was documented using Gel DOC system (Alpha imager, Alpha Innotech, USA).

3.9 ANALYSIS OF MOLECULAR MARKER DATA

3.9.1 Band scoring

All the images of resolved PCR products were taken. Clear and reproducible bands were taken for scoring. Binary scoring was carried out by assigning "1" for the presence of a specific band and "0" for the absence of a band. The data was entered in excel and was used as an input for cluster analysis and other statistical analysis.

3.9.2 Polymorphic Information Content (PIC)

After scoring of bands using molecular data, bands may be present or absent in the different isolates. If a particular band is present in some isolates and absent in some others, then the band is said to be polymorphic. According to the formula PIC = 2PiQi described by Tehrani *et al.* (2008); each individual SSR allele's PIC value was calculated. In this formulae, Pi is considered as the frequency of presence and Qi is the frequency of absence of a particular band. The expected heterozygosity (H_E) for all the SSR primers were also calculated using GenAlEx software 6.5 (Peakall and Smouse, 2006).

3.9.3 Frequency based analysis

The genetic differentiation among 5 populations based on geographical location of *P. colocasiae* was assessed by estimating F_{ST} and Nm values. F_{ST} is the degree of gene differentiation among populations in terms of allele frequencies and Nm is the estimated gene flow value. The gene flow value can be calculated by Nm=0.5(1− Gst)/Gst. This analysis was performed using GenAlEx software 6.5 (Peakall and Smouse, 2006).

3.9.4 Population genetic diversity analysis

Based on the genotypes present in a population, the genetic diversity of each population can be calculated. The 47 *P. colocasiae* isolates were grouped in to five populations based on their geographical locations. Population A contains the *P. colocasiae* isolates collected from Kerala; population B: isolates collected at fine spatial scale; population C: isolates collected from Andhra Pradesh; population D: isolates collected from Odisha; population E: isolates collected from Assam. The expected heterozygosity (Nei's gene diversity, H), Observed number of alleles (N_A), effective allele numbers (N_E), number of private alleles and Shannon's diversity index (I) were calculated in GenAlEx software 6.5 (Peakall and Smouse, 2006).

3.9.5 Tests for Hardy-Weinberg Equilibrium

Using ARLEQUIN software version 3.5 (Excoffier and Lischer, 2010), the significant deviations from Hardy–Weinberg equilibrium (HWE) at each locus were calculated. The chi- square test for HWE was also performed with 1,000,000 steps in the Markov chain.

3.9.6 Cluster analysis

Using 10 SSR primer combinations, the genetic relatedness of 47 *P. colocasiae* isolates were estimated based on the Nei and Li distance (Nei and Li, 1979). The genotypes were clustered using Treecon software 1.3b8 (Van de Peer and De Wachter, 1994), based on an UPGMA clustering algorithm. The

cophenetic correlation coefficient was calculated between the original distance matrix and the constructed dendrogram. The Mantel test (Mantel, 1967) was carried out to detect the goodness-of-fit of the cluster analysis of the matrix on which it was based (1000 permutations). This test was performed using GenAlEx software 6.5 (Peakall and Smouse, 2006).

3.9.7 AMOVA

Using GenAlEx software version 6.5 (Peakall and Smouse, 2006); PhiPT-values based analysis of molecular variance (AMOVA) were performed. AMOVA analysis will generate the degrees of freedom (df) value among the populations and within populations. The percentage of variation within and among the populations were estimated using AMOVA. Pair- wise PhiPT- values between two populations were calculated in order to estimate the degree of genetic differentiation between the populations.

3.9.8 Principal Coordinate Analysis (PCoA)

2 Dimensional- Principal Coordinate analysis (PCoA), a multivariate analysis was done using GenAlEx software 6.5 (Peakall and Smouse, 2006). This analysis will group the isolates in two coordinates and we can estimate the divergence exhibited by them.

3.9.9 Factorial analysis

Factorial analysis was performed with genetic distance matrix created using DARwin software ver. 6.0.014 (Perrier *et al.*, 2003). The factorial analysis was done to offer an overall representation of the diversity in this study by analysing the genetic relationships among the genotypes.

3.9.10 Model based population structure

The SSR genotyping results were used to perform population structure analysis for the 47 isolates under an admixed model using the STRUCTURE program version 2.3.4 (Pritchard *et al.*, 2003). The varieties under diverse populations were classified as admixture or pure based on the membership fractions. Alpha value was estimated to determine the presence of admixture. An

alpha value close to zero implies that most isolates included in the study are from separate populations. But most of the isolates of populations are considered to be admixed, when the alpha value is greater than 1.

RESULTS

4. RESULTS

The results of the study on "Genetic diversity analysis of *Phytophthora colocasiae* using SSR markers" carried out at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2016-2017 are presented in this chapter.

4.1 SAMPLE COLLECTION

The leaf samples with typical leaf blight symptom of water soaked lesion were collected from taro (*Colocasiae esculenta* L. Schott.) growing fields at ICAR - Central Tuber Crops Research Institute (ICAR- CTCRI), Sreekariyam, Thiruvananthapuram for the isolation of *P. colocasiae* on a random basis. Infected leaf samples with large, water soaked, round to irregular, dark brown spots on the lamina were collected on a random basis. With the severity of the disease, yellow to red liquid drops developed on the spot which was visible during early morning but later appears as solid and brown in colour (Plate 2). Leaves having infection at its earlier stage were appropriate for pathogen isolation and found not successful with decayed leaf samples. In certain instance, fresh symptoms of the infection were produced artificially on healthy taro leaves with decayed and highly infected samples to facilitate successful pathogen isolation.

4.1.1 Isolation of *Phytophthora colocasiae*

A total of 15 *P. colocasiae* isolates were isolated from the leaf blight infected taro fields of ICAR- Central Tuber Crops Research Institute, Thiruvananthapuram, India during this course of study. The pathogens were isolated from multiple leaf blight lesions of a single infected taro leaf and also from different infected leaves of the same plant. Surface sterilization of infected leaf bits using 0.8% mercuric chloride is found to be more effective in comparison with 1% sodium hypochlorate solution. The number of bacterial contaminants on the Potato Dextrose Agar (PDA) isolation plates were effectively reduced with the addition of ampicillin (100 mg l^{-1}) (Plate 3). All the fifteen isolates were initially detected as

Plate 1. Leaf blight infected taro plant

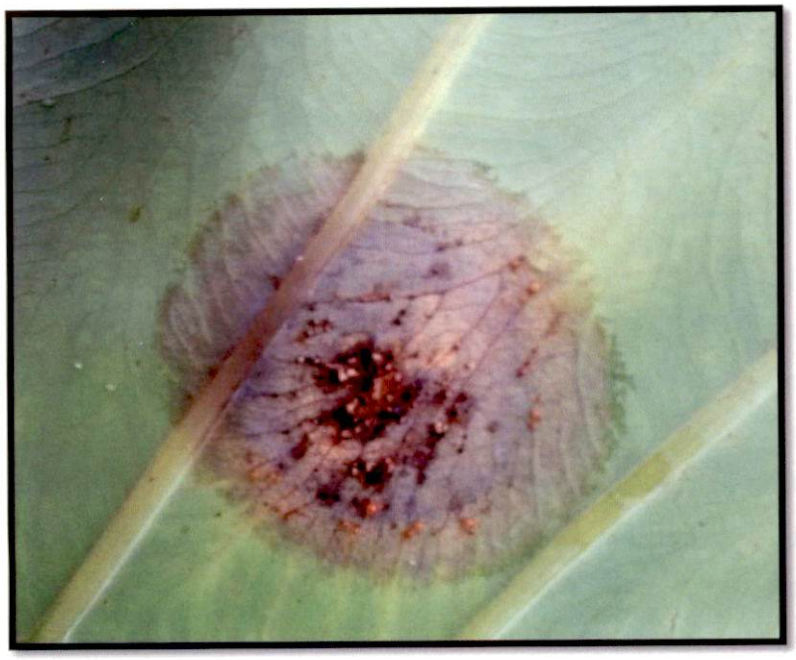

Plate 2. Brown colour liquid ooze out from the leaf blight lesion

Phytophthora colocasiae through its mycelial and sporangial characters. *P. colocasiae* isolates were able to produce ellipsoid, ovoid or fusiform semipapillate sporangia that are caducous and with a medium pedicel (3·5–10µm) (Plate 4). Mycelium was aseptate, hyaline and the colony was submerged or fluffy in its growth with whitish or dull white colour in carrot agar medium. The characteristics of the pathogen were similar to the descriptions given by Waterhouse (1963) and Lebot *et al.* (2003).

4.1.2 Revival and subculture of *P. colocasiae*isolates

Thirty-two *P. colocasiae* isolates collected from various Indian states conserved in the ICAR-CTCRI repository, were revived by inoculating the cultures artificially on leaf blight susceptible variety 'Sree Kiran'. Pathogens were re-isolated from the infectious leaf tissue after 2-3 days on ampicillin (100 mg l^{-1}) supplemented PDA medium. *P. colocasiae* isolates were then sub-cultured in carrot agar (CA) medium. Each isolate was stored long term in glycerol (50 %) at −20 °C. Carrot agar (CA) slants were prepared and inoculated with *P. colocasiae* and kept in the dark at 18 °C for conducting the further studies.

4.2 CULTURE CHARACTERIZATION

Growth characteristics, sporangial production and colony morphology were studied on six different media: Czapek dox agar (CDA), Carrot agar (CA), Potato dextrose agar (PDA), Carrot potato agar (CPA) Yeast peptone glycerol (YPG) and V8 juice agar showed considerable morphological differences between *P. colocasiae* isolates. They produced diverse colony morphology with respect to different culture media used (Plate 5). The growth rate and sporangial production of the pathogen on different media were recorded 7 days after inoculation and the results were presented in Table 3. The results revealed that among all the media tested, maximum growth of the pathogen was recorded on Carrot Agar (84 mm), followed by Czapek Dox agar (CDA)medium (76 mm), whereas minimum growth of the pathogen was recorded on PDA medium (34 mm). Hence the pathogen was maintained on carrot agar medium for conducting further studies. Moreover, the

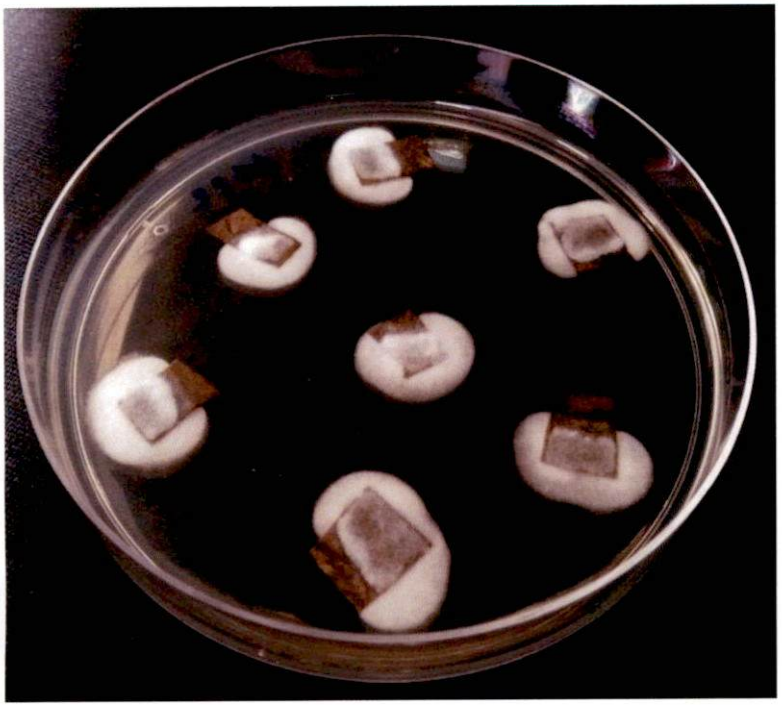

Plate 3. *Phytophthora colocasiae* colony isolated from infected leaf tissues on PDA media

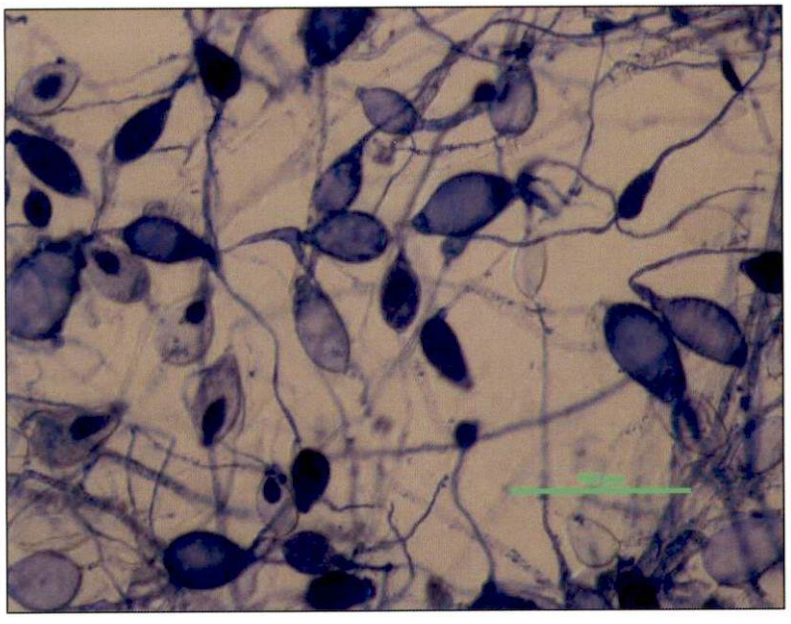

Plate 4. Sporangial morphology of *P. colocasiae*

isolates from different geographical areas exhibited considerable morphological variations in PDA medium (Plate 6). Variations observed in the growth rates include isolates with cottony morphology had a greater growth rate compared to other *P. colocasiae* isolates. For the proper growth and sporulation of *P. colocasiae* isolates, the optimum temperature was found to be 27-30°C (Figure 1).

Table 3. Radial growth rate and *in vitro* sporulation of *P. colocasiae* on different culture media

Culture media	Radial growth rate (mm)	Sporulation ($x10^6$ sporangia/ml)
CA	84.67 ± 03^{d}	18^{d}
CPA	74.06 ± 03^{c}	9^{c}
CDA	76.24 ± 00^{c}	8^{c}
PDA	34.18 ± 03^{a}	1^{b}
V8 Juice agar	54.74 ± 03^{b}	17^{d}
YPG agar	38.12 ± 03^{a}	0^{a}

Means with the same letter are not significantly different according to Duncan multiple range test (P=0.05)

4.2.1 Sporangial morphology

The studies of sporangial morphology under light microscope revealed that they were hyaline, ovoid and semipapillate in shape. There were considerable differences in the sporangial morphology of different *P. colocasiae* isolates. The dimensions (length and breadth) of sporangia were measured, at 40 X magnification for each isolates and were tabulated (Table 6). The average length of the *P. colocasiae* sporangia was found to be 32.73-52.78 µm and breadth to be 18.06-32.12 µm.

64

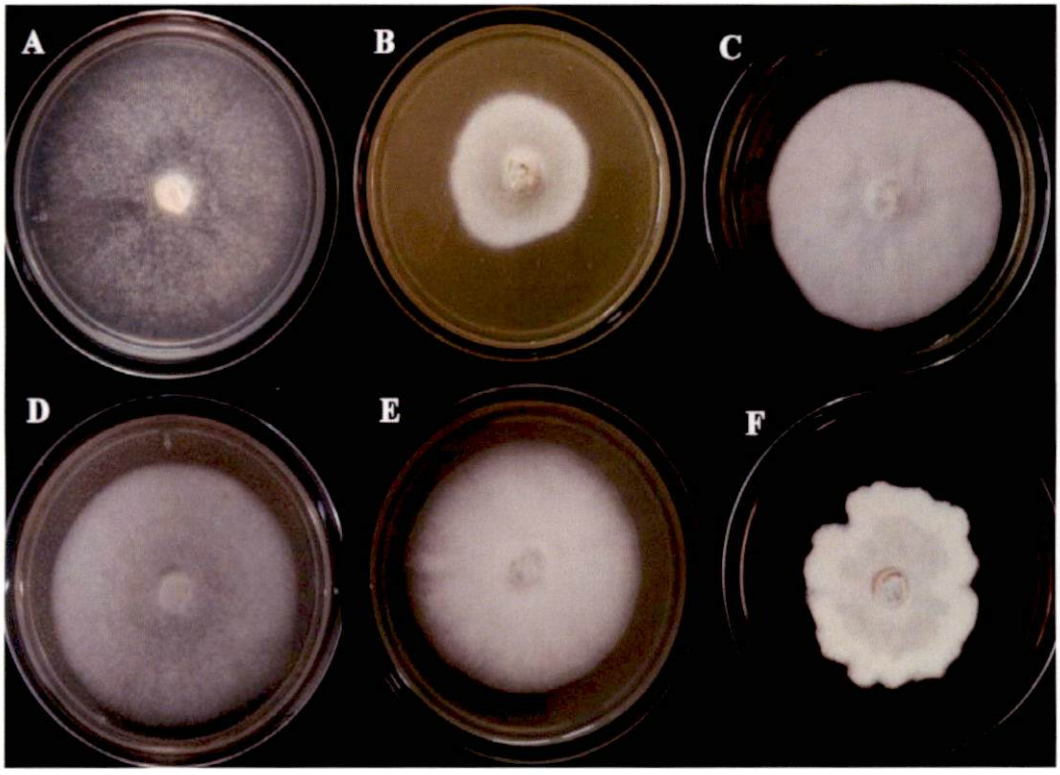

Plate 5. Morphological variation exhibited by *P. colocasiae* in six different culture media

Culture media shown are A: Czapek Dox agar; B: Vegetable juice agar; C: Potato dextrose agar; D: Carrot agar; E: Carrot potato agar; F: Yeast peptone glycerol agar

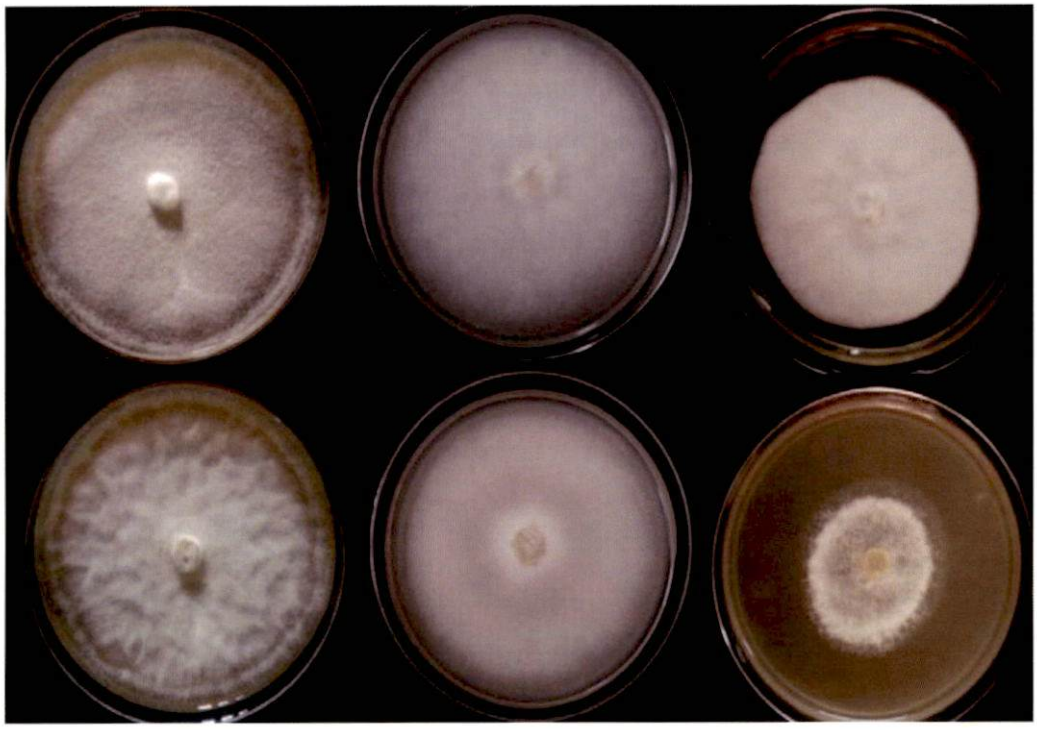

Plate 6. Morphological variation exhibited by *P. colocasiae* isolates in PDA medium

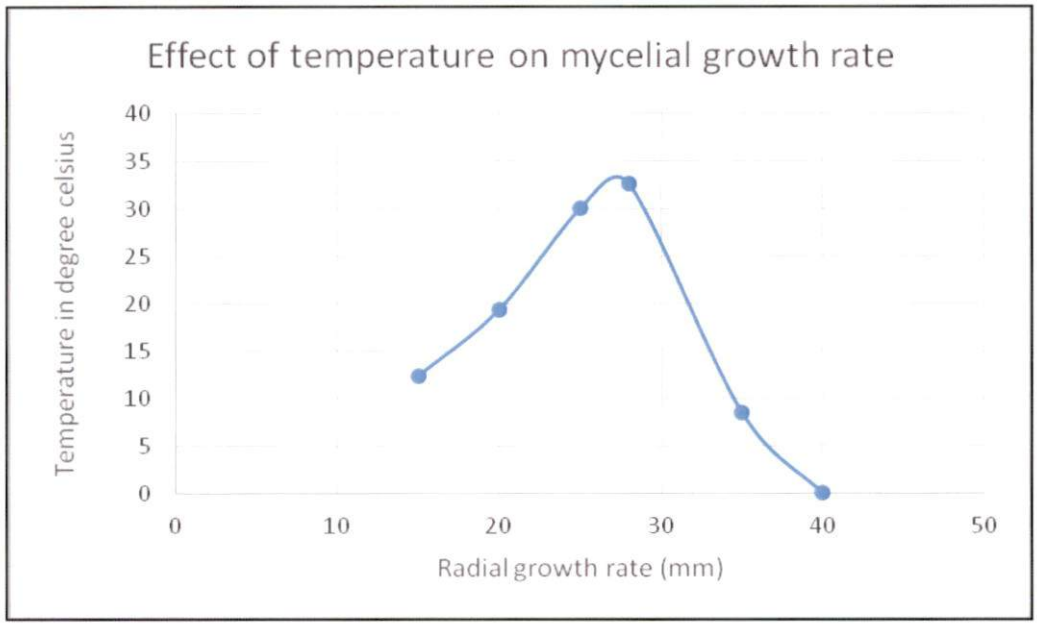

Figure 1. Effect of temperature on growth rate of *P. colocasiae* in PDA medium

4.2.2 Virulence assay

The isolates were able to produce lesions 2-3 days after inoculation (d. a. i.) on healthy taro leaves. The inoculation point at the beginning, appeared as yellow to brown water- soaked lesion which became dark brown on the disease progression (Plate 8). A considerable difference was observed in the lesion diameter produced by isolates of different geographical origins (Table 4, Figure 2). On the control leaf disc, no lesions were observed after 4 days of incubation. The pathogens were re-isolated from all the developed lesions according to Koch's postulate.

Table 4. Culture characteristics of selected *P. colocasiae* isolates used in this study

Isolate code	Colony appearance on PDA	Sporangial dimensions (µm)		Growth rate on PDA (mm day^{-1})	Lesion diameter 4 d.a.i (cm)
		Length	Breadth		
PC31	Flat with concentric rings	52.73± 0.12^{f}	29.98±0.15^{e}	4.82±0.00^{c}	2.96±0.05^{d}
PC21	Irregular pattern	47.08±0.15^{d}	22.18±0.08^{c}	4.86±0.03^{c}	2.17±0.05^{b}
PC20	Uniform without pattern	32.73±0.12^{a}	18.06±0.17^{a}	4.02±0.03^{a}	3.25±0.05^{e}
PC13	Plain	49.14±0.18^{e}	19.98±0.12^{b}	5.06±0.00^{d}	3.96±0.10^{f}
PC37	Cottony	40.98±0.11^{b}	29.78±0.19^{e}	5.74±0.03^{f}	2.72±0.05^{c}
PC11	Cottony with concentric rings	42.01±0.15^{c}	32.06±0.13^{f}	5.76±0.03^{f}	1.67±0.05^{a}
PC40	Plain with irregular concentric rings	52.78±0.13^{f}	27.39±0.08^{d}	5.38±0.03^{e}	4.35±0.05^{h}

Values are the means ± SE of three replicates. Within columns, means followed by same letter do not differ significantly according to Duncan's multiple range test at $P \leq 0.05$.

d.a.i. = days after inoculation

4.3 STANDARDIZATION OF DNA ISOLATION PROTOCOL

Kit based and manual methods for DNA isolation were carried out for the isolation of genomic DNA from 47 *P. colocasiae* isolates. Plate 9 shows the DNA profile on agarose gel using kit method: Genomic DNA Purification Kit

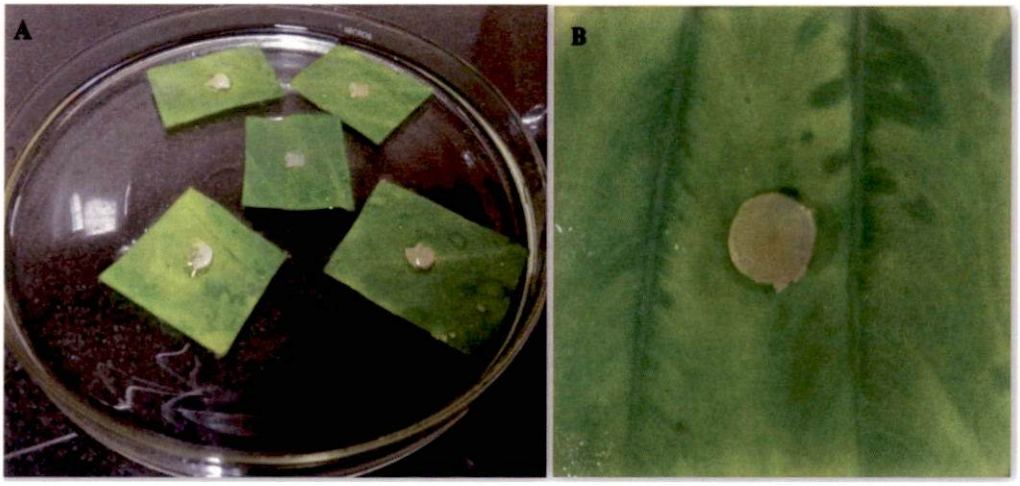

Plate 7. Pathogenicity assay on leaf blight-susceptible variety "Sree kiran" leaf discs

A: Floating leaf disc method of pathogenicity assay; B: Control leaf disc 4 d.a.i

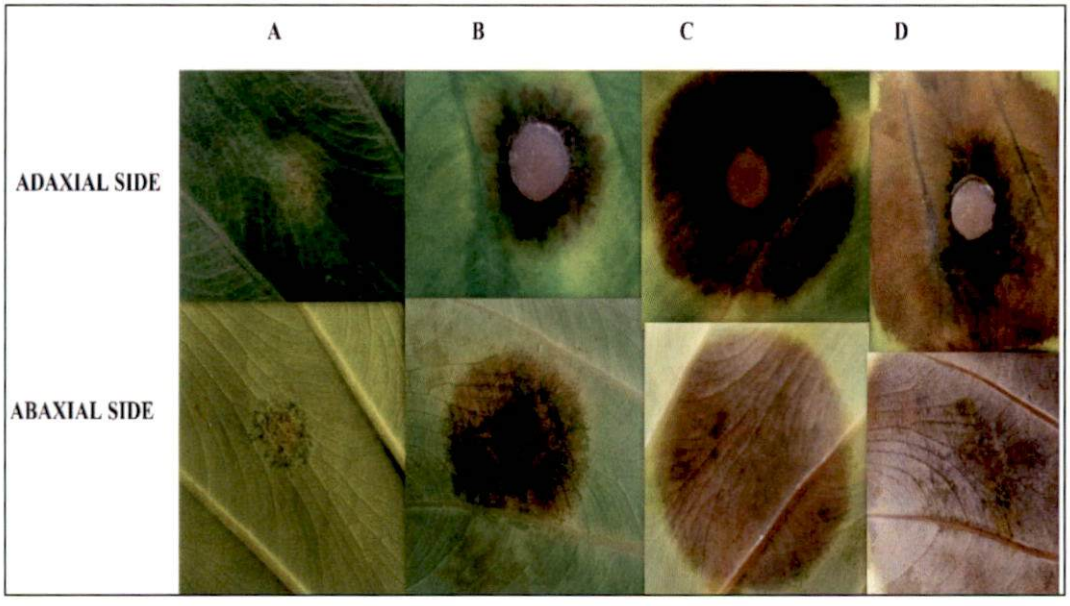

Plate 8. Disease progression on taro leaf discs

A: 1 d.a.i; B: 2 d.a.i; C: 3 d.a.i; D: 4 d.a.i

(Thermo Fisher Scientific, *Fermentas, EU*), using Cooke and Duncan DNA isolation method for *Phytophthora* (1997) and modified Cooke and Duncan DNA isolation method. DNA isolated using commercial kit based method was found to be intact and gave good spectrophotometric readings. A_{260}/A_{280}ratio between 1.86-2.04, depicts a high quality DNA, with no traces of RNA and protein contaminations. The Cooke and Duncan DNA isolation method for *Phytophthora* (1997) gave sheared and low intensity bands for many of the isolates after resolving it in 1% agarose gel. The spectrophotometric readings indicate a higher amount of RNA contamination in the extracted genomic DNA. Moreover, DNA extraction from samples with higher mycelial age is found to be difficult with this method. Certain modifications in the Cooke and Duncan method (Including an additional chloroform: iso amyl alcohol (24:1) step and the final precipitation of DNA using 3 M sodium acetate and ice cold isopropanol (1:3)) made an impact in the concentration of DNA. Concentration was increased several fold as compared to that of conventional Cooke and Duncan method of DNA extraction (Figure 3). Spectrophotometric reading A_{260}/A_{280}ratio between 1.82- 1.88, depicts a high quality DNA, with no traces of RNA and protein contaminations. The concentration and purity of the DNA isolated using three methods were compared and tabulated (Table 5, Plate 9). This modified Cooke and Duncan method was found to be suitable for isolating genomic DNA from samples with any mycelial age. So modified Cooke and Duncan method of genomic DNA extraction method was used for the isolation of genomic DNA from the 47 *P. colocasiae* isolates used in the present study.

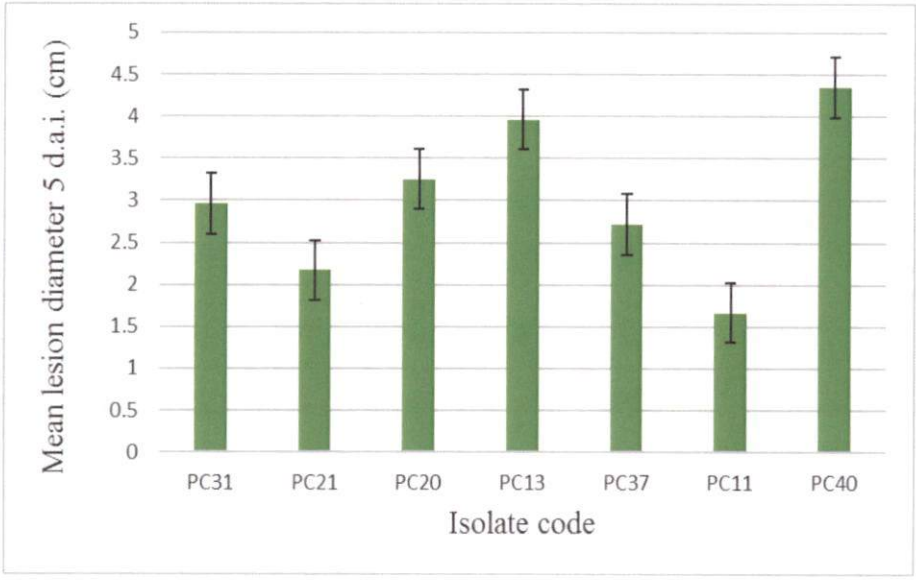

Figure 2. Mean lesion diameter measured on taro leaf disc 5 d.a.i. with selected *P. colocasiae* isolates

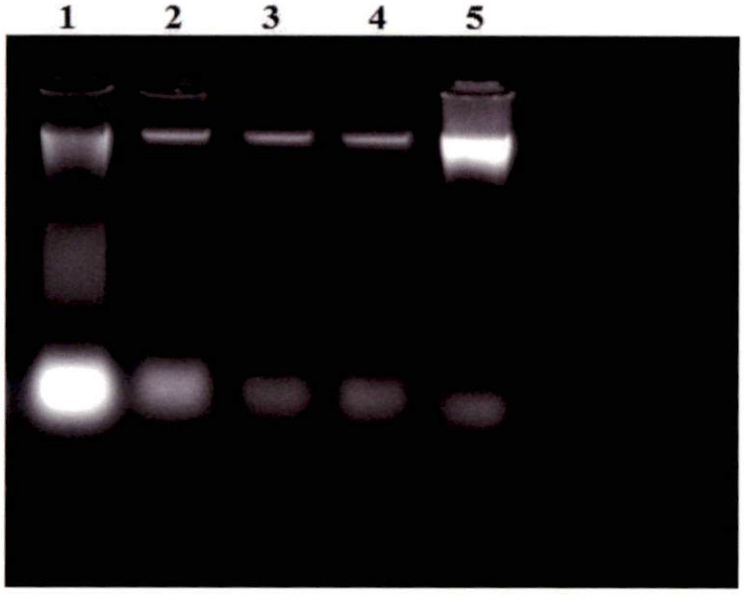

Plate 9. Genomic DNA from *P. colocasiae* isolate CTCRIPC25

Lane 1: DNA extracted using Cooke and Duncan DNA isolation method for *Phytophthora;* Lane 2, 3, 4: DNA extracted from *P. colocasiae* having different mycelial age using modified Cooke and Duncan method; Lane 5: Using Genomic DNA Purification Kit.

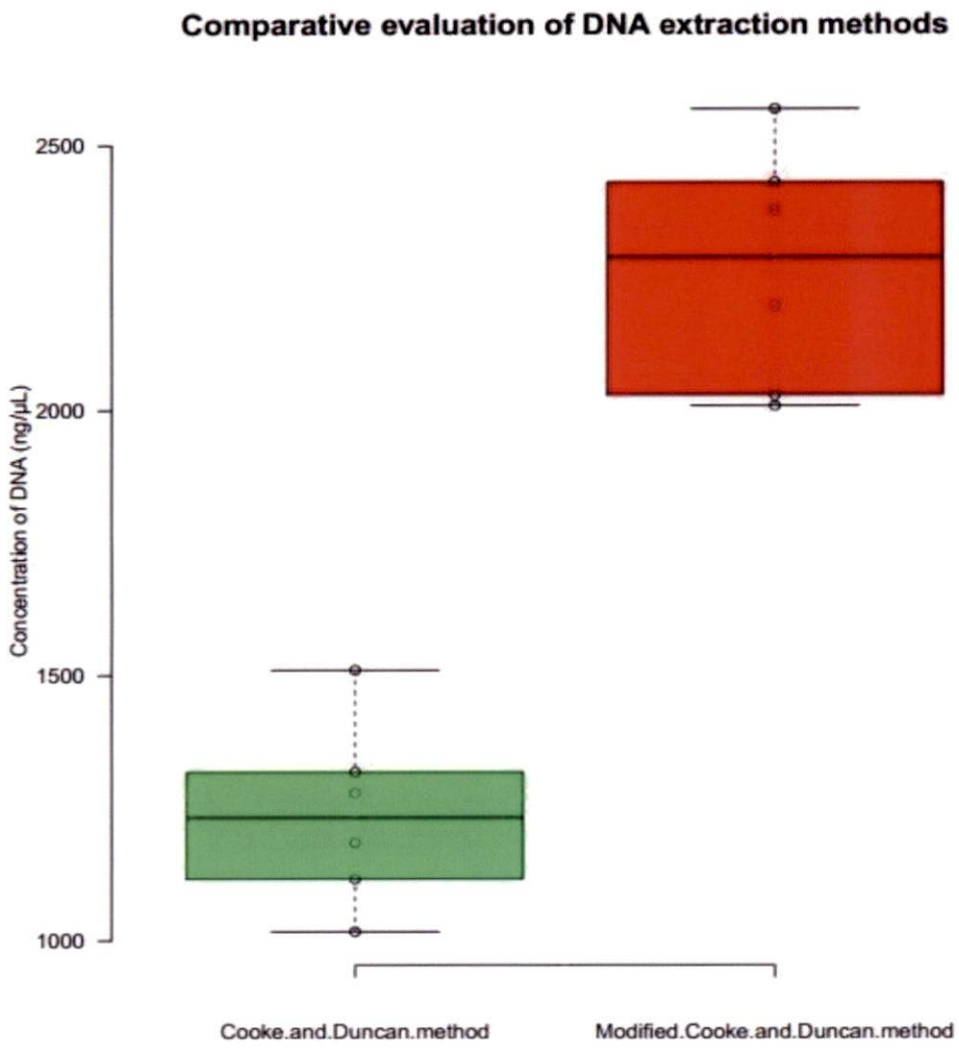

Figure 3. Comparative evaluation of DNA extraction methods using boxplot

Table 5. DNA concentration of 80 mg tissue sampled for three methodologies, revealed by Nanodrop spectrophotometer

Method	Sample	DNA yield (ng μl^{-1})	$A_{260/280}$	$A_{260/230}$
Modified Cooke and Duncan DNA isolation method	PC20	2568	1.87	2.18
	PC21	2431	1.83	2.09
	PC22	2009	1.88	2.2
	PC24	2379	1.84	2.12
	PC25	2198	1.85	2.16
	PC26	2029	1.82	2.04
Cooke and Duncan DNA isolation method for *Phytophthora* (1997)	PC20	1510	2.14	2.34
	PC21	1280	1.99	2.28
	PC22	1117	2.12	2.39
	PC24	1319	1.98	2.37
	PC25	1186	2.01	2.32
	PC26	1017	1.93	2.29
Genomic DNA Purification Kit (Thermo Fisher Scientific, *Fermentas, EU*)	PC20	2219	1.86	2.16
	PC21	1899	1.84	2.18
	PC22	1798	1.85	2.14
	PC24	1956	1.83	2.10
	PC25	1834	1.84	2.12
	PC26	1798	1.81	2.03

4.4 PCR ANALYSIS WITH SPECIES SPECIFIC PRIMERS

All the 47 *P. colocasiae* isolates were confirmed at molecular level by amplifying its genomic region using primer pairs (Nath *et al.*, 2014) corresponding to *RAS*-related protein gene *Ypt1*which is a monomeric GTP-binding protein, indispensable in vesicle transport and secretion. The primer pairs had successfully

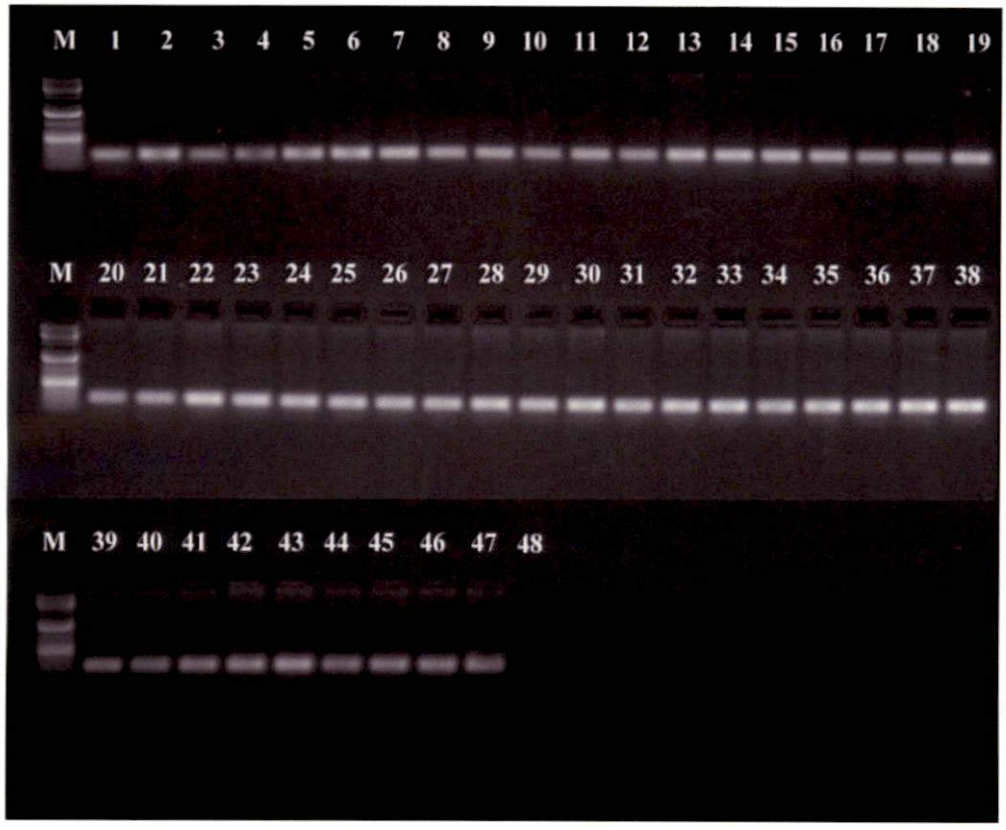

Plate 10. Species specificity of amplification with the *P. colocasiae* species-specific primer pair

Lane M: 1 Kb plus DNA ladder; Lane 1-47: *P. colocasiae*; Lane 48: buffer control

amplified all the 47 *P. colocasiae* isolates under the optimized PCR conditions. When resolved on 1.5% agarose gel, they yielded an amplicon of size 206bp (Plate 10).

4.5 GENETIC DIVERSITY FROM FINE SPATIAL SCALE

Five *P. colocasiae* isolates were collected from multiple leaf blight lesions on a single taro leaf, in order to check the variability exhibited by them (Plate 11). The diversity exhibited by *P.colocasiae* isolates obtained from fine spatial scale were assessed by ITS characterization and sequencing.

4.5.1 ITS characterization

The ITS region including the 5.8 S rRNA gene was amplified successfully to check the hyper variability exhibited by the pathogen. All the five isolates yielded ~750 bp product when amplified with universal ITS primers (ITS1 - ITS4) (Plate 12). The PCR products were successfully purified with GeneJET gel extraction kit (Thermo Scientific, USA). Upon ITS sequence analysis, 97-99% nucleotide sequence similarity was observed with each other and with the other *P. colocasiae* sequences available at NCBI GenBank. Sequence alignment using Clustal W software exposed significant SNPs in the 5 isolates collected at fine spatial scale (Figure 4). These nucleotide sequences were submitted under accession numbers KY432684.1, KY432685.1, KY432681.1, KY432682.1 and KY432683.1 in the NCBI GenBank.

4.6 PRIMER SCREENING AND STANDARDIZING ANNEALING TEMPERATURE (Ta)

A total of 25 SSR primers were taken for determining its amplifying capability and the annealing temperature. DNA samples of two isolates (CTCRIPC25 and CTCRIPC28) were taken for this screening purpose. Plates 13 and 14 shows the gel profile of screening 6 primers at different temperatures. A total of 15 primers were eliminated from further analysis as they gave unclear or no

Plate 11. Taro leaf showing multiple leaf blight lesions

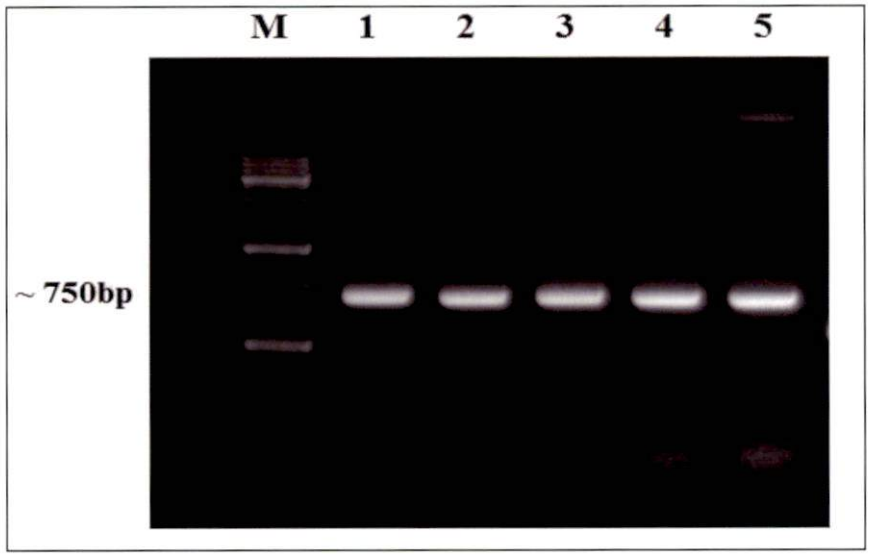

Plate 12. 1.5% agarose gel profile of five *P. colocasiae* ITS amplification

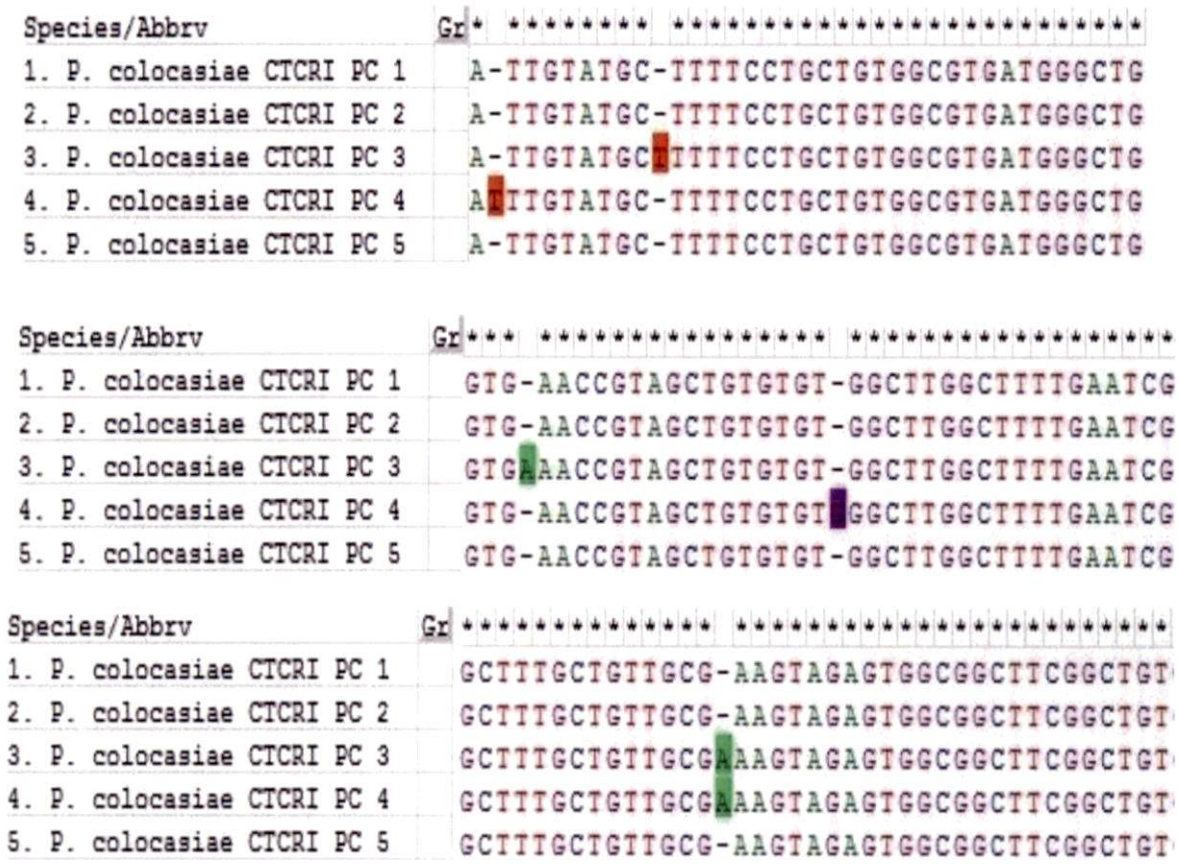

Figure 4. Aligned DNA sequences of ITS region of selected five *Phytophthora colocasiae* isolates.

bands. The ten primers selected for SSR analysis, its annealing temperatures are given in the table 6.

Table 6. List of selected 10 SSR primers along with their annealing temperatures

Forward primers	Reverse primers	Annealing temperature	SSRs
S19F GGGAGCTACTCGAGGTGGTG	S20R TCGTCTCAATCTCKGACTGA	54.5°C	$(AGC)_6$
S14F GACGCMSYYGAGTGGAAAG	S15R ATTTKGSACAGATACCGACG	56.4°C	$(AAG)_{15}$
R10F GGAGATGACGGAAGATGACG	R11R CCATCGAARTACATSACACGA	55.5°C	$(AAGCC)_4$; $(AGG)_9$; $(AAG)_7$
R1F GYGGCGGTGGCTACAGYG	R3R CTGCTGYTGCTGGTTGAAAG	54.5°C	$(ACC)_4$; $(ACC)_5$; $(ACC)_4$
I7F GHGTGGGCGAGTACTCCAAG	I8R AAGCTGGCTATRWACACTGCCG	55.9°C	$(AG)_9$
I9F GCATYGGGTCGTTCCTGTA	I10R AGHGTGCAGTACAGACCCGC	53.5°C	$(AAG)_{11}$
R15F CCGGAGCGCGTGGA	R16R GGTAGTTGAGCGGCTTCTTG	57.4°C	$(CCG)_6$ $(AGC)_{14}$
R4F YATGGGWGGCATGATGAAC	R5R AGGACCAGGAGATGGAGGAC	55.5°C	$(AGC)_4$; $(AGC)_{12}$; $(AGC)_4$
S12F GGAGGCCGAGTCGGARTA	S13R TAYTCCGACTCGGCCTCC	53.5°C	$(AGC)_{14}$
R16F CAAGAAGCCGCTCAACTACC	R17R TAACGGATCAGCTCTTGCTG	56.4°C	$(ATC)_4$; $(AGG)_8$

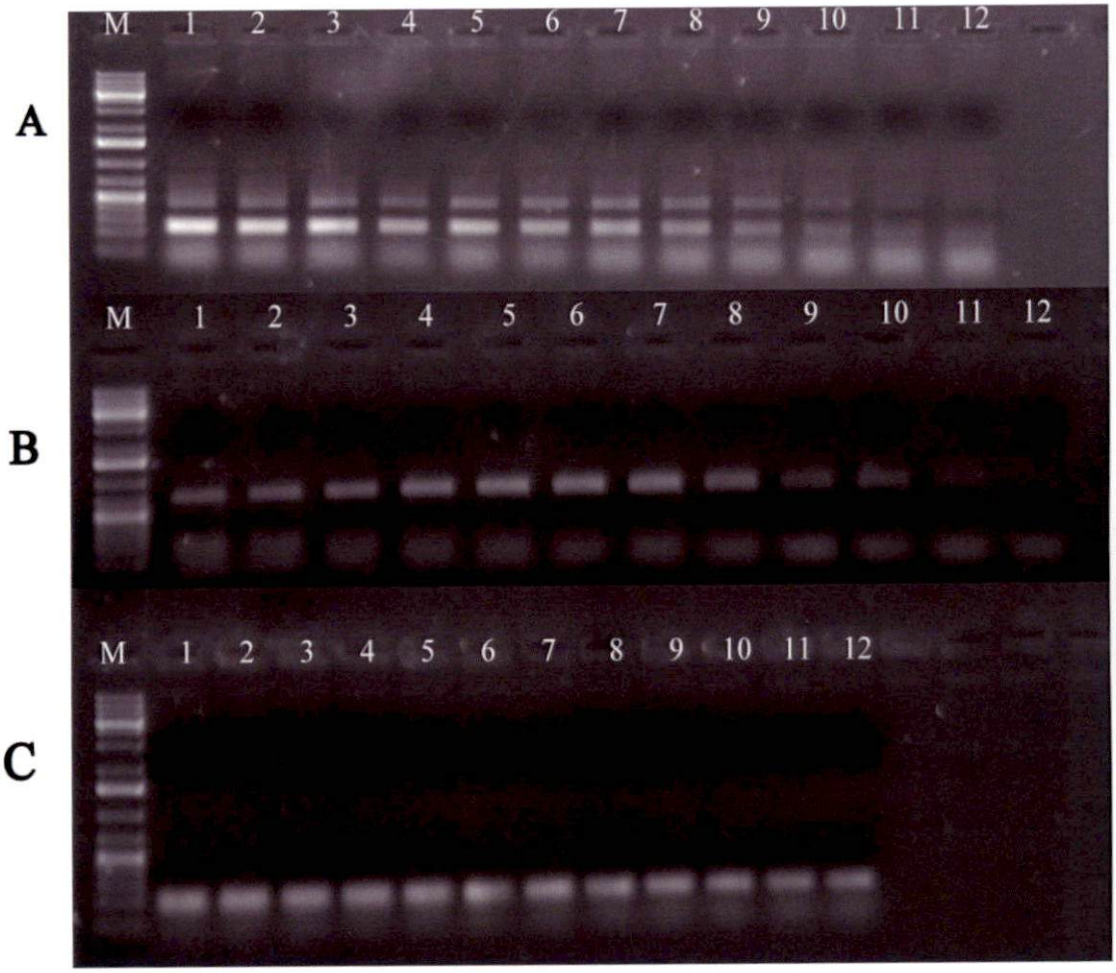

Plate 13. Gel profile of CTCRIPC 25 using 3 SSR primers

Primers:

A – R1F/R3R B – R4F/R5R C – I11F/I12R

Temperature:

1– 50.2° C; 2 – 50.5° C; 3- 51.3 ° C; 4- 52.5 ° C; 5- 53.5° C;

6- 54.5° C; 7- 55.5 ° C; 8- 56.4 ° C; 9- 57.4 ° C; 10- 58.4 ° C;

11- 59.7 ° C; 12- 60 ° C

DNA:

CTCRIPC25

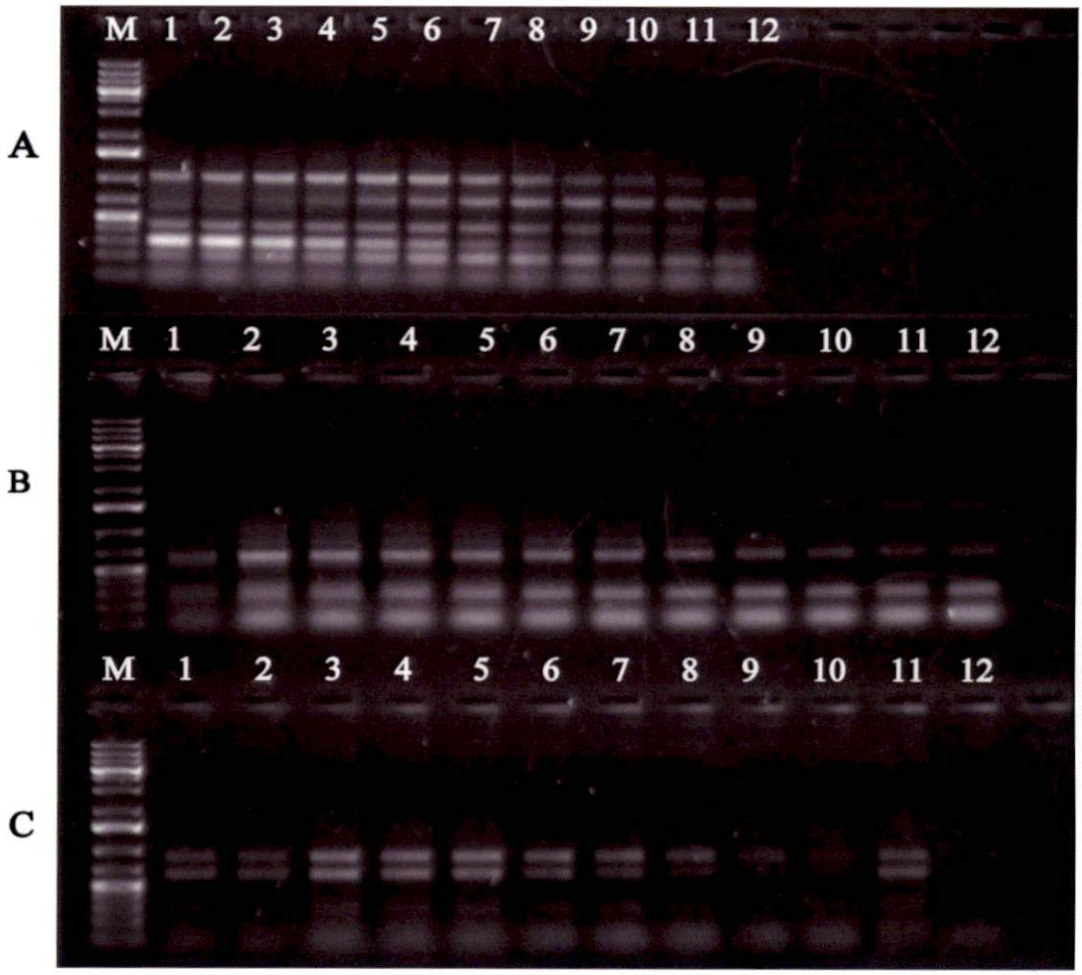

Plate 14. Gel profile of CTCRIPC28 using 3 SSR primers

PrImers:

A – R10F/R11R B – R15F/R16R C – R16F/R17R

Temperature:

1– 50.2° C; 2 – 50.5° C; 3- 51.3 ° C; 4- 52.5 ° C; 5- 53.5° C; 6- 54.5° C;

7- 55.5 ° C; 8- 56.4 ° C; 9- 57.4 ° C; 10- 58.4 ° C; 11- 59.7 ° C;

12- 60 ° C.

DNA:

CTCRIPC28

4.7 SSR ANALYSIS

Genetic diversity analysis of 47 *Phytophthora colocasiae* cultures collected from different geographical locations of India were analysed using reported 10 SSR markers (Schena *et al.*, 2008). After performing PCR using the selected SSR primers at specific PCR conditions and determined annealing temperatures. The amplicons were resolved in 2% agarose gel with 1 kb plus DNA ladder (Fermentas). The bands obtained for selected SSR primers are shown in Plate 15-18.

4.8ANALYSIS OF MOLECULAR MARKER DATA

Ten SSR loci have been evaluated in 47 isolates of *P. colocasiae* from different geographical locations of India. We detected 54 alleles with an average of five alleles per locus. The number of alleles per locus varied from 2 for the primer combination S12F/S13R to 9 for the primer combinations S14F/S15R and I9F/110R. Seven markers detected at least five alleles and only three amplified less than five alleles.

4.8.1 Polymorphic Information Content (PIC)

The polymorphic bands obtained with each primer pair was scored for their presence (1) or absence (0). According to the formula PIC = 2PiQi described by Tehrani *et al.* (2008); each individual SSR allele's PIC value was calculated. In this formulae, Pi is considered as the frequency of presence and Qi is the frequency of absence of a particular band. PIC values for all the polymorphic bands produced by a primer pair were averaged to calculate PIC value for a primer pair. (Table 7). The Polymorphism Information Content (PIC) values was found to be in the range of 0.359 to 0.819 with an average of 0.601. Three SSR primers revealed PIC values higher than 0.70 and are I9F/ I10R, R10F/R11R and S14F/S15R. Among the 10 SSR primers, I9F-I10R exhibited higher PIC and expected heterozygosity with 0.819 and 0.822 respectively. Only two SSR primers exhibited a PIC value less than

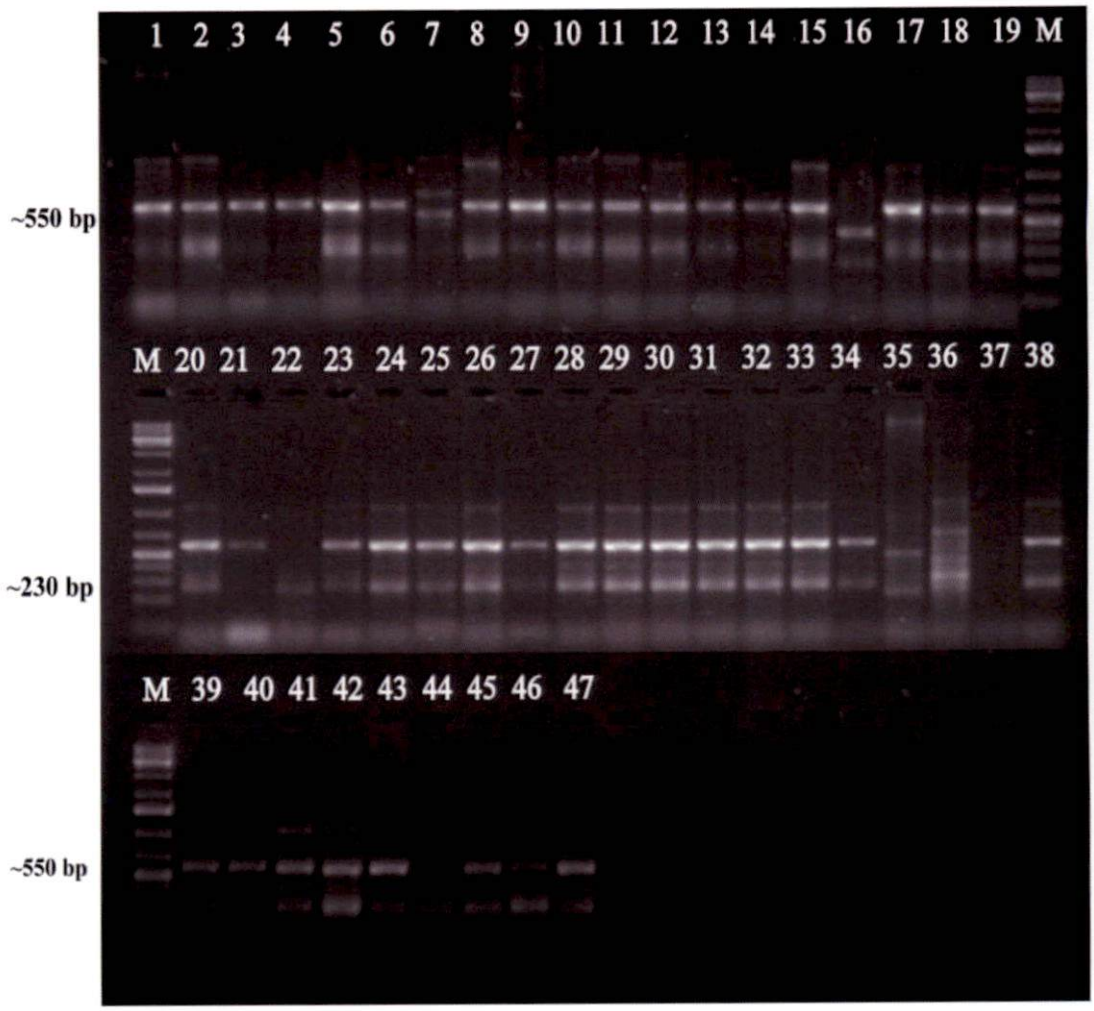

Plate 15. Gel profile of 47 isolates using the primer S19F/S20R

Lane M: 1 Kb plus DNA ladder

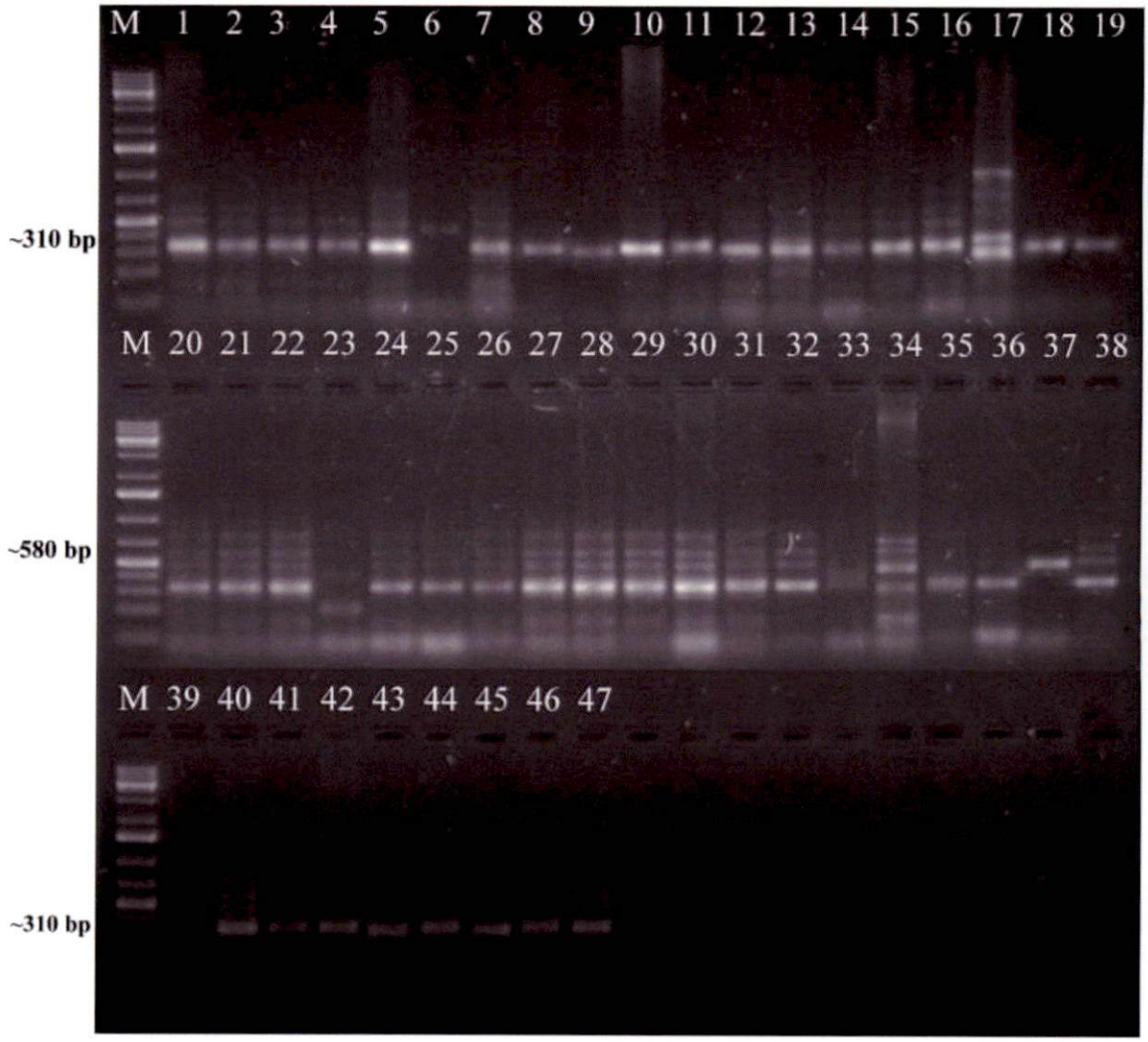

Plate 16. Gel profile of 47 isolates using the primer I7F/I8R

Lane M: 1 Kb plus DNA ladder

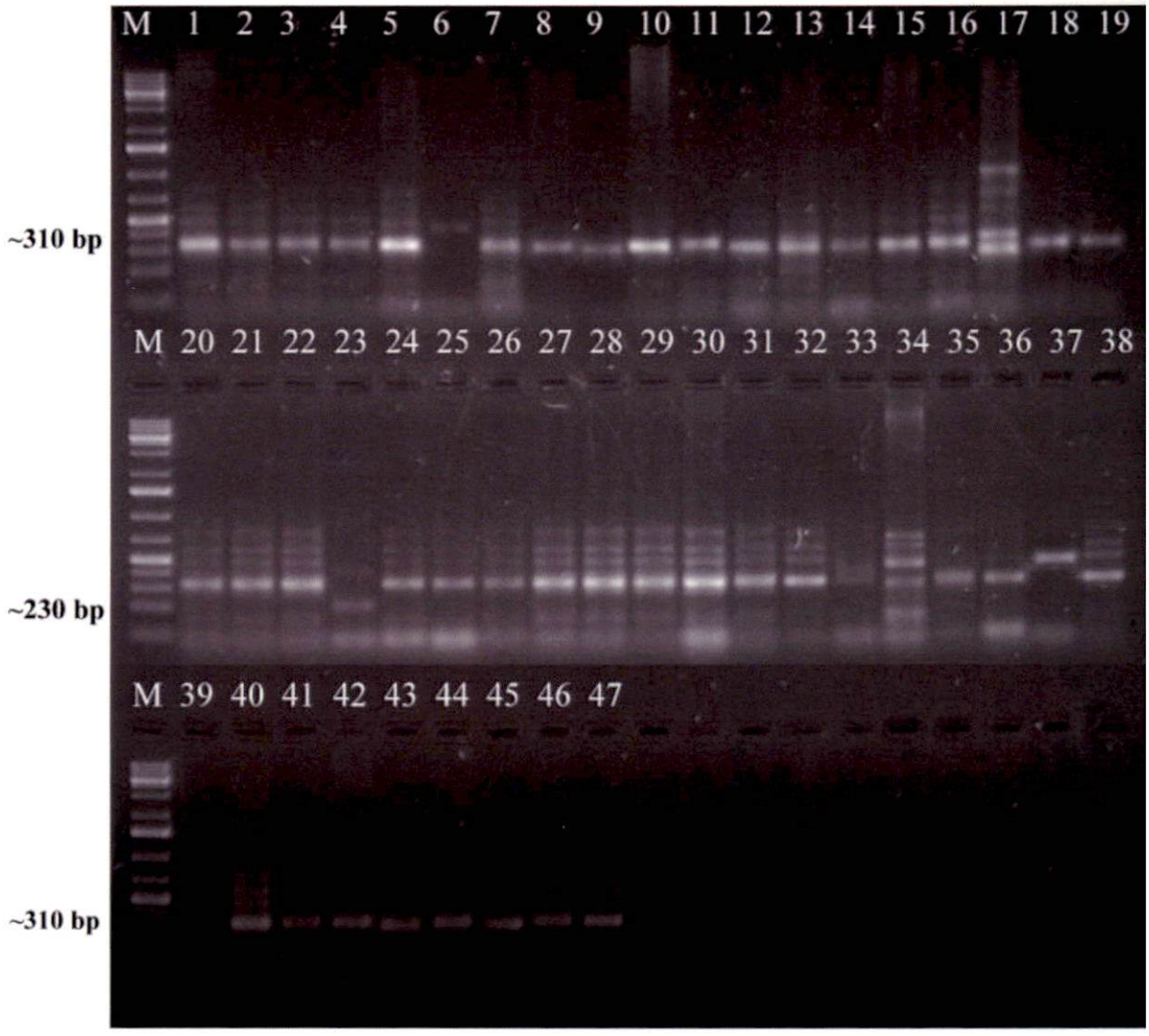

Plate 17. Gel profile of 47 isolates using the primer I9F/I10R

Lane M: 1 Kb plus DNA ladder

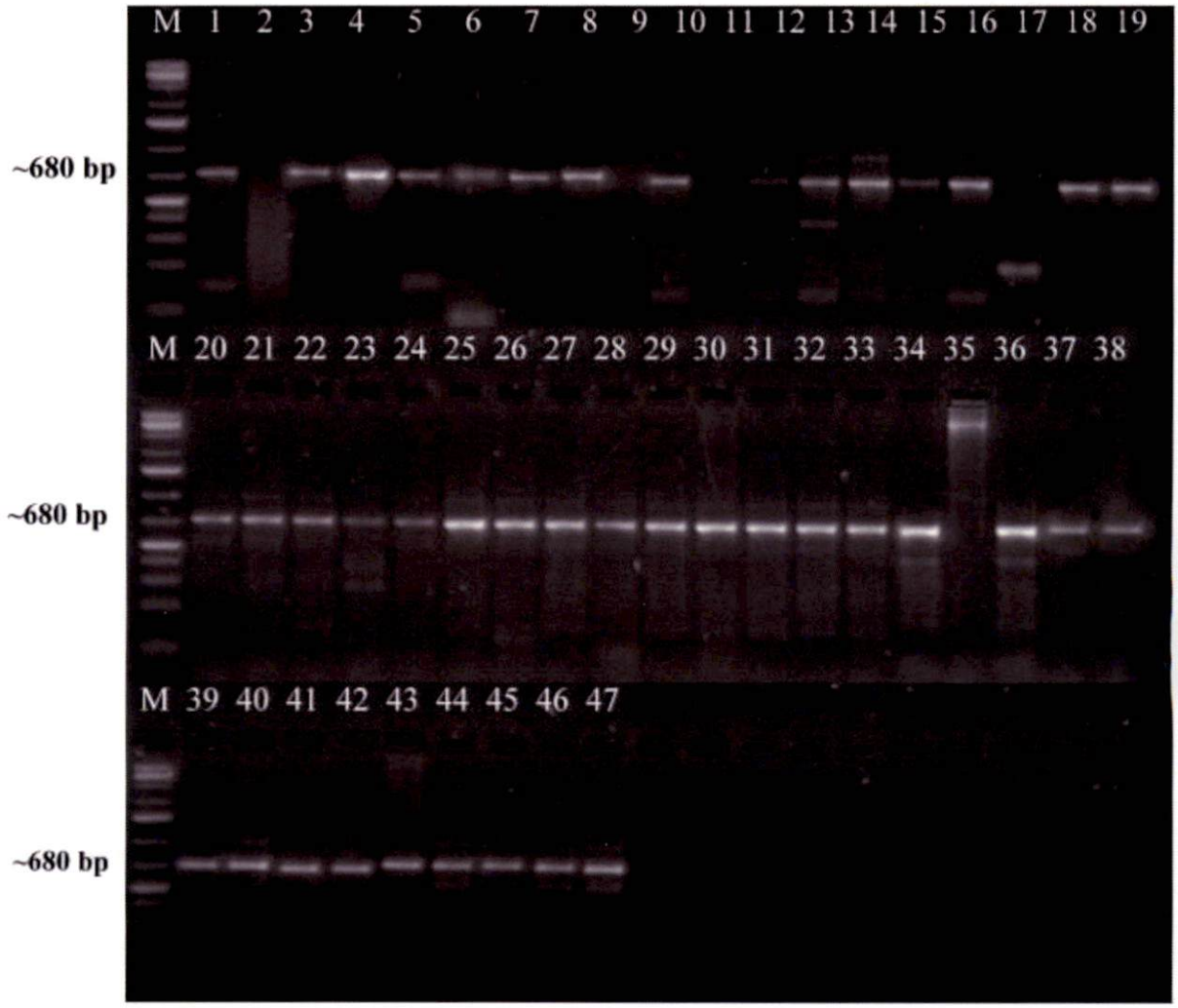

Plate 18. Gel profile of 47 isolates using the primer R4F/R5R

Lane M: 1 Kb plus DNA ladder

0.50 and are I7F/I8R and S12F/S13R. The SSR primer S12F/S13R exhibited least PIC and expected heterozygosity with 0.359 and 0.470 respectively.

Table 7. Polymorphic information content and expected heterozygosity were calculated for 10 SSR primers

Sl No.	SSR Primer	Repeat motif	TNB	NPB	Average PIC	Expected heterozygosity
1	I7F/ I8R	$(AG)_9$	3	3	0.393	0.507
2	I9F/ I10R	$(AAG)_{11}$	9	9	0.819	0.822
3	R1F/ R3R	$(ACC)_4$; $(ACC)_5$; $(ACC)_4$	7	7	0.528	0.840
4	R4F/R5R	$(AGC)_4$; $(AGC)_{12}$; $(AGC)_4$	7	7	0.605	0.606
5	R10F/R11R	$(AAGCC)_4$;$(AGG)_9$; $(AAG)_7$	5	5	0.767	0.799
6	R15F/R16R	$(CCG)_6$ $(AGC)_{14}$	6	6	0.509	0.593
7	S12F/S13R	$(AGC)_{14}$	2	2	0.359	0.470
8	S14F/S15R	$(AAG)_{15}$	9	9	0.778	0.807
9	S19F/S20R	$(AGC)_6$	6	6	0.667	0.719
10	R16F/R17R	$(ATC)_4$; $(AGG)_8$	3	3	0.592	0.666

TNB: total number of bands; NPB: number of polymorphic bands

4.8.2Frequency based analysis

The genetic differentiation among 5 populations based on geographical location of *P. colocasiae* was high and significant (Fst=0.4342, $P<0.001$). F_{ST} is the degree of gene differentiation among populations in terms of allele frequencies. The estimated gene flow, Nm (Nm=0.5(1− Gst)/Gst), was 0.3687. The results of

this analysis reveal that the genetic differentiation among populations throughout the entire distribution area is significant and that gene flow is restricted.

4.8.2.1 Population genetic diversity analysis

Based on the genotypes present in the populations, the genetic diversity of each population was calculated (Table 8). 47 isolates were grouped in to five populations based on their geographical locations. The expected heterozygosity (Nei's gene diversity, H) varied from 0.0 in population B to 0.44 in populations D and E. The calculated Shannon diversity index, varied from 0.0 in population B to 0.665 in population D. Observed number of alleles, effective allele numbers, number of private alleles and heterozygosity were generally low, but consistently higher in pop D (Figure 5).

Table 8. Analysis of divergence of genetic variation in populations of *P. colocasiae*

Population code	PPL	N_A	N_E	H	I
PopA	80%	2.20± 0.29	1.62±0.13	0.506±0.13	0.30±0.073
PopB	0%	1.00±0.00	1.0±0.00	0.0±0.00	0.0±0.00
PopC	90%	2.40±0.22	2.09±0.22	0.734±0.12	0.458±0.07
PopD	60%	1.60±0.34	1.36±0.27	0.44±0.13	0.284±0.08
PopE	90%	2.70±0.30	1.99±0.21	0.75±0.012	0.442±0.06

PPL: Percentage polymorphic loci; N_A: observed number of alleles; N_E: effective number of alleles; H: Nei's gene diversity; I: Shannon's information index;

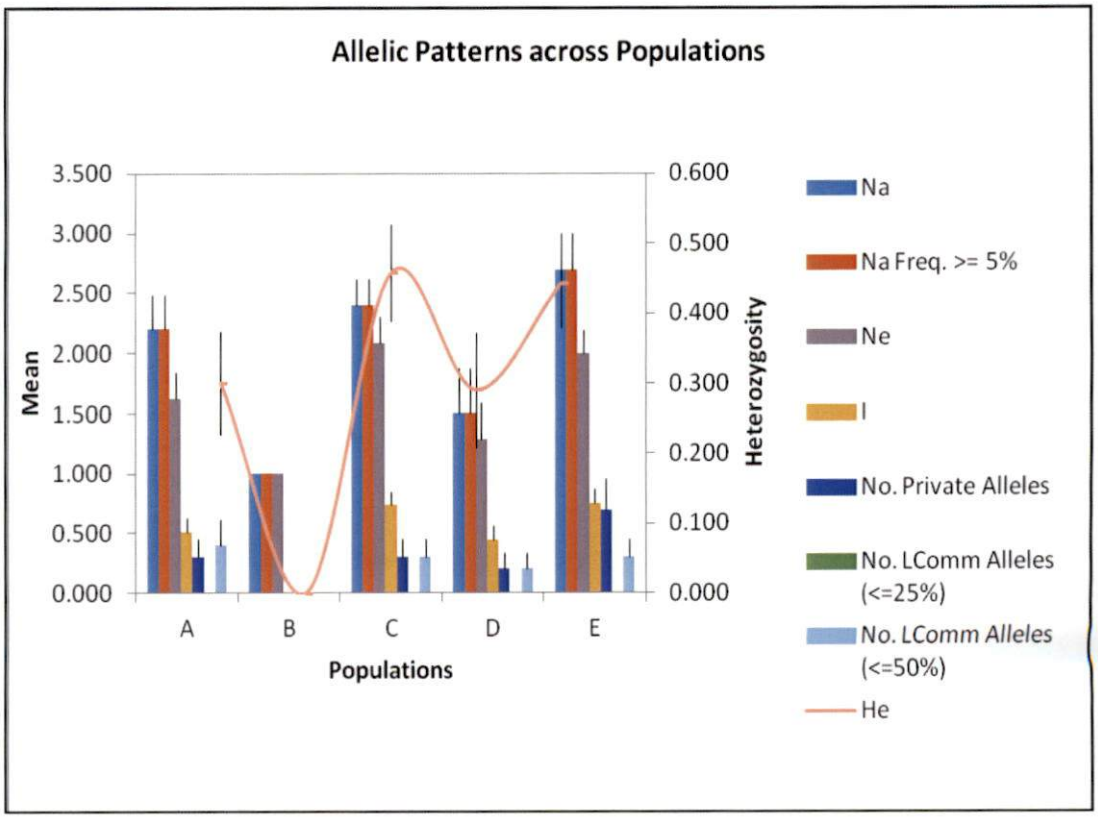

Figure 5. Estimated allelic patterns across 5 populations of *P. colocasiae*

4.8.2.2 Tests for Hardy-Weinberg equilibrium

Using ARLEQUIN software version 3.5 (Excoffier and Lischer, 2010), the significant deviations from Hardy–Weinberg equilibrium (HWE) at each locus were calculated. The chi- square test for HWE was also performed with 1,000,000 steps in the Markov chain. All the 10 SSR loci were found significantly deviated from HWE (P<0.001) (Table 9).

Table 9. Estimated chi- square and degrees of freedom (DF) for HWE in 10 studied loci

Locus	Chi- square	DF	Significance
Locus1	38.000	1	Significant ; P<0.001
Locus2	165.000	15	Significant; P<0.001
Locus3	180.000	15	Significant; P<0.001
Locus4	93.000	6	Significant; P<0.001
Locus5	56.000	3	Significant; P<0.001
Locus6	148.000	10	Significant; P<0.001
Locus7	144.000	10	Significant; P<0.001
Locus8	164.000	10	Significant; P<0.001
Locus9	230.000	15	Significant; P<0.001
Locus10	117.000	6	Significant; P<0.001

4.8.3 Cluster analysis

Using 10 SSR primer combinations, the genetic relatedness of 47 *P. colocasiae* isolates were estimated based on the Nei and Li distance (Nei and Li, 1979). The genotypes were clustered in to two major clusters (Figure 6) using

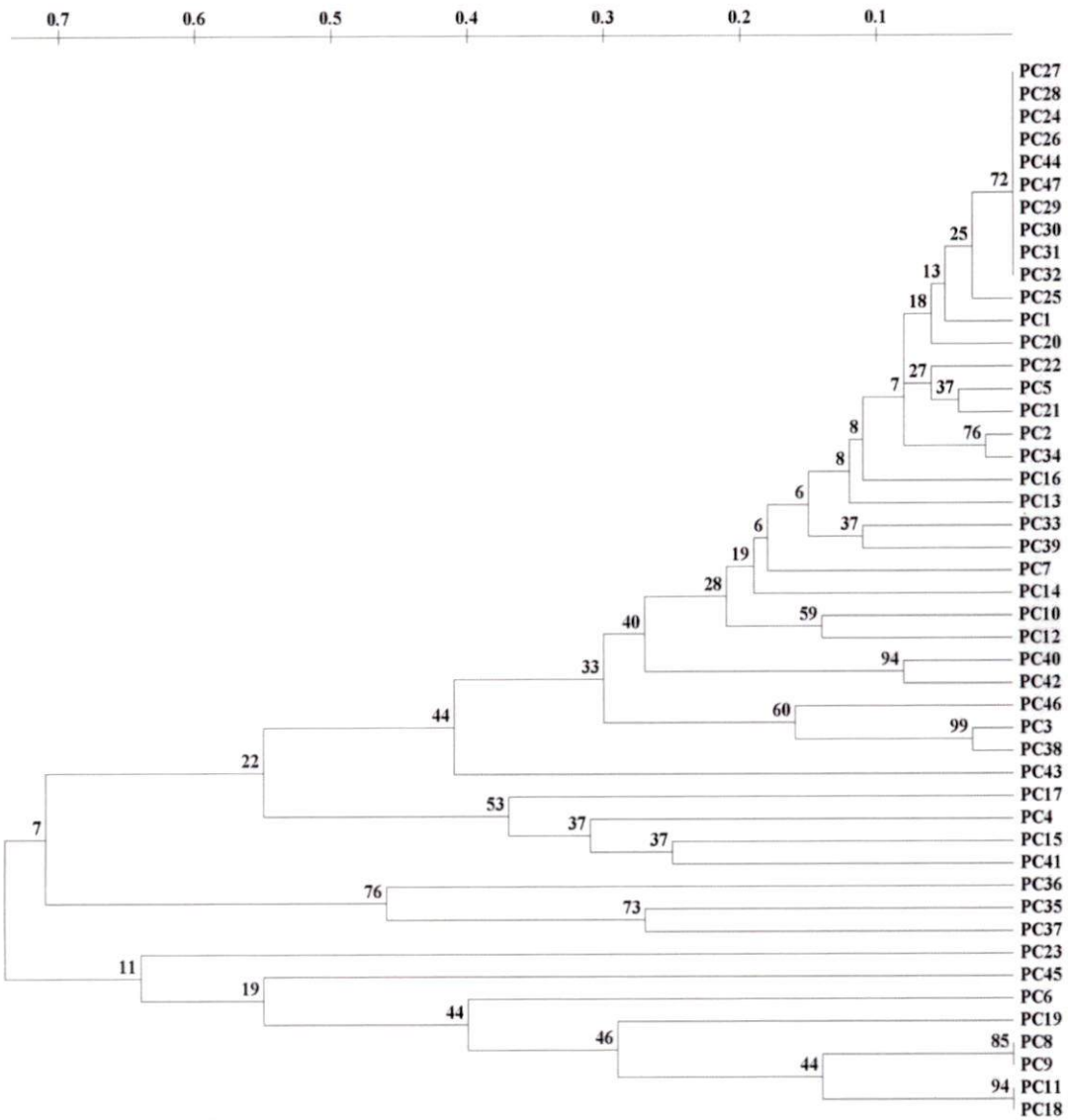

Figure 6. UPGMA dendrogram depicting genetic relatedness of *Phytophthora colocasiae* isolates based on SSR markers.

Numbers at the nodes represent bootstrap values (2000 replicates)

Treecon software 1.3b8 (Van de Peer and De Wachter, 1994), based on an UPGMA clustering algorithm. Cluster II formed the major group in 39isolates, while cluster I had 8 isolates. Cluster I contains 7 isolates collected from Odisha and one isolate from Assam. Isolates collected at fine spatial scale were grouped with higher similarity. The cophenetic correlation coefficient was calculated and found to be significant (r = 0.974) between the original distance matrix and the constructed dendrogram.

4.8.4 Mantle test

The Mantel test (Mantel, 1967) was carried out to detect the goodness-of-fit of the cluster analysis of the matrix on which it was based (1000 permutations). The correlation between clustering and genetic distance showed significance with a Rxy value of 0.782, P= 0.001.

4.8.5 AMOVA

In this study, the Analysis of molecular variance (AMOVA) based on PhiPT- values, depicted that a higher percentage (66%) of the *P. colocasiae* population's genetic diversity was allotted among population and only 34% within population (Figure 7). The degrees of freedom (df) value as per the AMOVA analysis was found to be 4 for among the populations and 42 within the populations (Table 10). The Phi PT value was found to be 0.661, *P* value of 0.001 and a Phi PR P-value of 0.003. These values were found to be less than 1 and this confirmed the credibility of AMOVA results. At all the two hierarchical levels, the genotypic diversity value (P) was found to be highly significant ($p < 0.001$). The pair-wise Phi PT values of two populations were compared and estimates the genetic distances between the populations. The highest differentiation was observed between Pop B and Pop D with a pair-wise Phi PT value of 0.933 and lowest differentiation was observed between Pop A and Pop B with a pair-wise Phi PT value of 0.100. There exist no or negligible genetic diversity between the ten *P. colocasiae* isolates belonging to population B.

Table 10. Analysis of molecular variance (AMOVA) of 47 isolates of *Phytophthora colocasiae* using SSR markers

Source	df	SS	MS	Est.Var.	%	*P*
Among populations	4	59.216	14.804	1.363	40%	<0.001
Within populations	42	87.167	2.075	2.075	60%	<0.001
Total	46	146.383		3.438	100%	<0.001

df= degrees of freedom, SS= sum of squares, MS= mean squares, Est. var.= estimate of variance, %= percentage of total variation, P- value is based on 1000 permutations.

4.8.6 Principal Coordinate analysis (PCoA)

Principal Coordinate analysis (PCoA), a multivariate analysis was performed and are generally in agreement with UPGMA cluster analysis results. The first coordinate axis accounted for 25.33 % of the total variance. It is observed that the second and third coordinates of the PCoA produced 17.35 % and 9.63 % respectively of the total variation using 10 SSR markers among 47 genotypes of *P. colocasiae* collected from different parts of India (Figure 8).

4.8.7 Factorial analysis

Factorial analysis was performed with genetic distance matrix created using DARwin software ver. 6.0.014 (Perrier *et al.*, 2003). The factorial analysis was done to offer an overall representation of the diversity in this study by analysing the genetic relationships among the genotypes. Factorial analysis (Figure 9) supported the groupings by dendrogram and showed a cluster of *P. colocasiae* isolates collected from Kerala in to a separate group. Interestingly, a close agreement was observed between the results arising from factorial analysis and STRUCTURE.

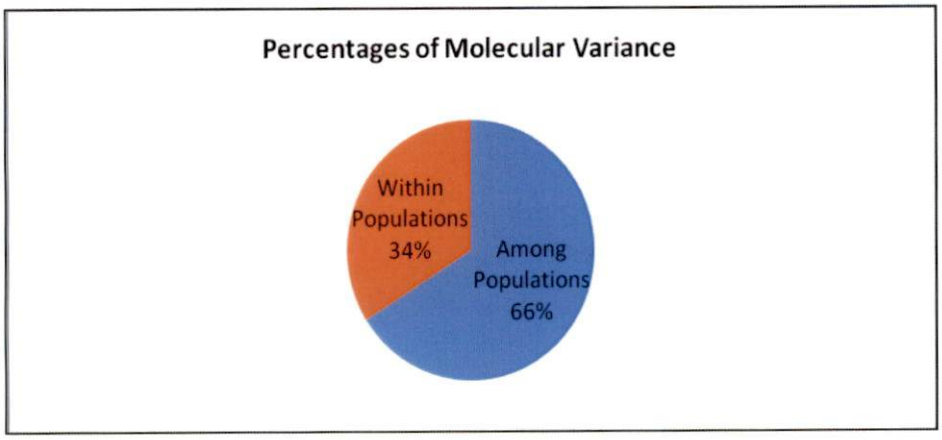

Figure 7. Analysis of molecular variance (AMOVA) of 47 *P. colocasiae* isolates based on populations obtained by geographical distribution of the pathogen.

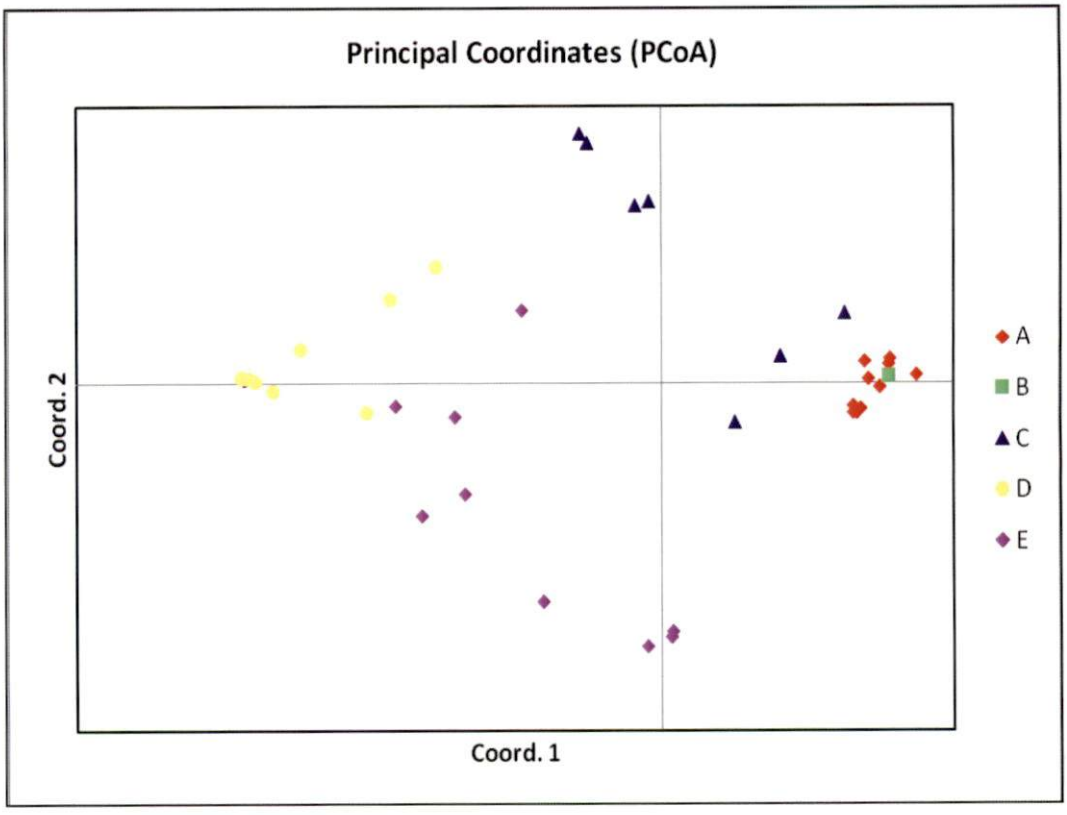

Figure 8. Principal coordinate analysis (PCoA) of 47 *P. colocasiae* isolates based on geographical locations.

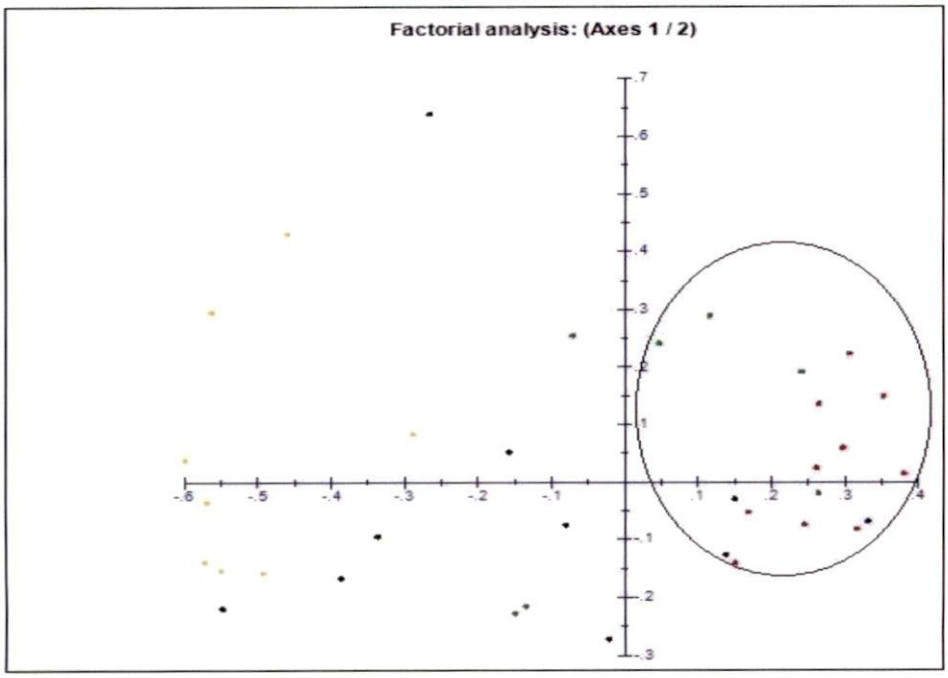

Figure 9. Factorial analysis of the 47 *P. colocasiae* isolates based on 10 SSR markers.

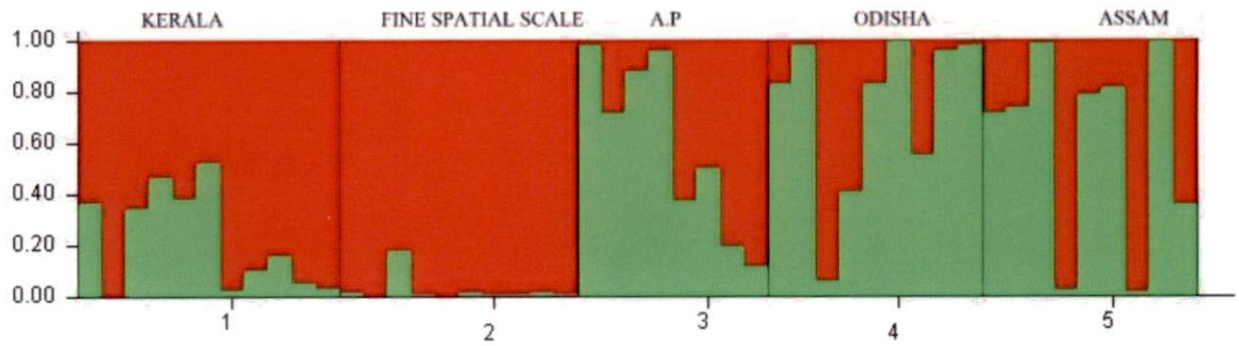

Figure 10. Population structure of 47 *P. colocasiae* isolates inferred by STRUCTURE 2.3.4 are represented by two colours.

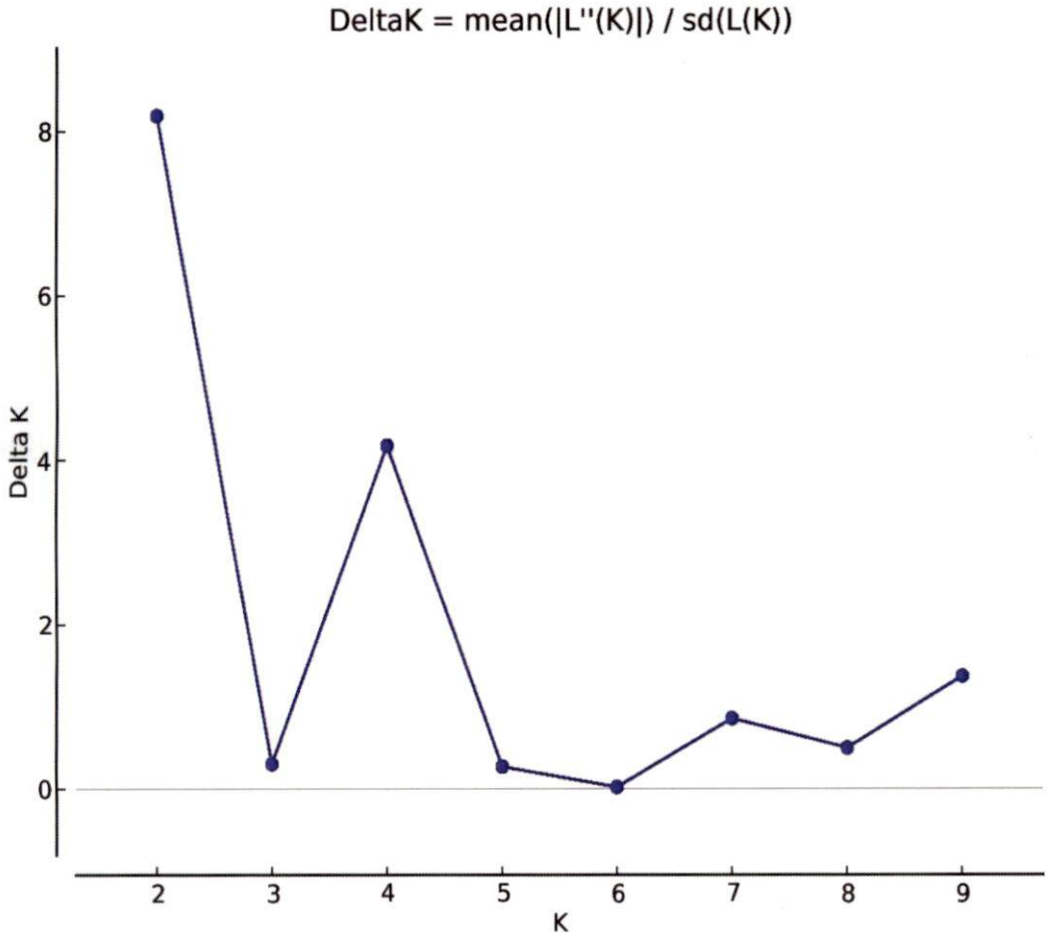

Figure 11. Estimation of population using LnP(D) derived Δk for k from 1 to10. ΔK reached its maximum value when K = 2, following the *ad-hoc* method

4.8.8 Model based population structure

The SSR genotyping results were used to perform population structure analysis for the 47 isolates under an admixed model using the STRUCTURE program version 2.3.4. Population structure divided the isolates into 2 populations (Figure 10). The isolates gained probability value greater than 0.80 was found to be pure and those with lower values than 0.80 as an admixture. Only few individuals were admixed in the present study as the value of alpha is found to be relatively lower ($\alpha = 0.0836$). The structure harvester computed best value of K at 2 (Figure 11).

DISCUSSION

5. DISCUSSION

Taro (*Colocasia esculenta* (L.) Schott), of the monocotyledon family *Araceae* is widely cultivated in the humid tropical areas of the world. Among the diverse diseases and pests of taro, leaf blight caused by the oomycete pathogen *Phytophthora colocasiae* is considered as the most destructive. This pathogen causes extensive foliar damage and serious postharvest decay of corms. In all the taro growing areas of India, leaf blight has become the major constrain for taro production, causing 30-50% yield loss (Misra *et al.*, 2008). For the development of good breeding strategies and appropriate utilization of plant resistance mechanisms to manage the disease effectively, an immense knowledge on the pathogen diversity is prerequisite. Pathogen populations having higher genetic diversity are more easily get adapted to extreme environments. *P. colocasiae* is a heterothallic oomycete, which requires both its mating types (A1 and A2) for oospore generation. Sexual reproduction can happen more easily in fields were both mating types are present and can result in production of highly virulent pathogens (Goodwin *et al.*, 1995). Compared to asexually produced sporangia, oospores can withstand more unfavorable situations and survive for longer periods.

Phenotypic variations among *P. colocasiae* isolates were studied much before in terms of growth characteristics, colony morphology, growth rate, sporulation and degree of virulence (Nath *et al.*, 2014, Padmaja *et al.*, 2014) *P. colocasiae* isolates having distant geographical origins, had exhibited higher rate of genetic diversity (Lebot *et al.*, 2003; Mishra *et al.*, 2010; Nath *et al.*, 2013). Only limited number of reports were available on the diversity studies of *P. colocasiae* using various molecular markers. The genetic diversity analysis of *P. colocasiae* isolates obtained from Pacific region and south east Asia were performed earlier by Lebot *et al.* (2003) using RAPD and isozyme markers. They predicted the presence of diverse *P. colocasiae* strains and significant variation were also observed among the isolates having distant geographical origins. Similarly, considerable genetic diversity was observed among the 14 *P. colocasiae* isolates obtained from different

geographical areas of India by Mishra *et al.* (2011. Nath *et al.* (2013) studied the hypervariability of *P. colocasiae* isolates at fine spatial scale, through ITS characterization and sequencing. But to date no work have been published regarding the genetic variability exhibited by *P. colocasiae* isolates using SSR markers. The objective of present study was to unravel the morphological and molecular diversity among the *P. colocasiae* isolates collected from diverse geographical areas in India and fine spatial scale.

5.1 Morphological characterization

Phenotypic characteristics showed considerable variation in all the parameters analyzed *viz.*, colony morphology, growth rate in different culture media, variation in sporangial morphology and virulence of the pathogen. Depending on the culture medium in which they were grown, *P. colocasiae* isolates exhibited a diverse colony morphology, growth rate and sporulation. Moreover, isolates from different geographical areas have shown considerable morphological variation when cultured in PDA medium. Phenotypically *P. colocasiae* isolates were characterized earlier by Nath *et al.* (2014) and he had elucidated nine morphological groups based on variability shown in PDA. In this study, eight morphotypes were observed when the isolates grown in PDA medium. The present study could find more morphotypes than a prior study by Mishra *et al.* (2011). The possible reasons behind this contradictory results could be due to the higher number of pathogenic isolates used in the present study from diverse geographical areas of India.

Comparative evaluation of growth characteristics and sporulation of *P. colocasiae* isolates in different culture media showed variable morphological characteristics, sporangial dimensions and growth rates. Among the various nutrient media tested, carrot agar was found to be most suitable for mycelial and sporangial production of *P. colocasiae*. Palomar *et al.* (1999) and Djeugap *et al.* (2009) studied different media for growth rate of *P. colocasiae*, and they reported V-8 and V-6 juice agar respectively as the suitable medium for growth. While Padmaja *et al.* (2014) reported carrot agar as the suitable growth media for large

scale sporangial production of *P. colocasiae*. The size, shape and dimensions of the observed *P. colocasiae* sporangia were found to be exactly similar to the descriptions of Palomar *et al.* (1999) and Omane *et al.* (2012). Microscopic examinations revealed that they were hyaline, ovoid and semipapillate in shape which is a characteristic feature of *Phytophthora* species.

Later, a study on effect of temperature on mycelial growth rate of *P. colocasiae* revealed an interesting result. Isolates having cottony morphotype were able to withstand 35°C while other isolates grow at 28°C. Earlier studies by Nath *et al.* (2014) identified isolates with stellate morphology were also be able to withstand extreme temperatures. These results were found to be in close agreement with the results of prior study by Mishra *et al.* (2011). The only one exception is that in the present study for any of the isolates no growth was observed at 40°C. During diversity analysis using SSR markers these isolates were grouped together in to a separate cluster. Moreover, during virulence assay those isolates collected from Odisha with a cottony morphology exhibit comparatively higher degree of virulence.

Pathogenicity tests revealed that newly obtained isolates were found to be highly virulent and were producing severe infection on leaf discs in comparison with isolates obtained from ICAR- CTCRI repository. Granke *et al.* (2011) found that long term culture storage can results in reduction of pathogen's virulence property. While conducting the floating leaf disc method of pathogenicity assay, there exist a significant variation in the mean lesion diameter produced by the tested isolates. The higher genetic diversity present in the *P. colocasiae* isolates were reflected here with the variability in the lesion diameter. Mishra *et al.* (2011) had stated that *P. colocasiae* isolates obtained from Kerala to be less pathogenic. But they were found to be equally virulent as those isolates from other locations of India in the current study. Significantly equal degree of virulence was exhibited by *P. colocasiae* isolates collected at fine spatial scale.

5.2 Molecular characterization

The PCR based molecular marker techniques have become the powerful tools for characterization of pathogens at molecular level. Genomic DNA with relatively high quantity and quality is required for successful PCR amplifications. The DNA extraction protocol by Cooke and Duncan (1997) was found very efficient in case *Phytophthora* species in several previous studies. However, in this study, genomic DNA isolation from samples with higher mycelial age found to be difficult and yielded sheared DNA with low quantity. In spite of, high amount of polyphenols good quality un-sheared DNA was obtained while using commercial kits. High cost and low quantities of DNA obtained make commercial kits undesirable for this study which contains multiple isolations. Certain modifications were introduced in to Cooke and Duncan protocol in order to minimize DNA loss from the crude tissue extract. Often, secondary chemical reactions like oxidation in the initial crude tissue extract can result in loss of DNA yield. The standard method to extract nucleic acids by removing proteins is by extracting the crude tissue extract once with a 25:24:1 mixture of phenol, chloroform and isoamyl alcohol. An additional step of extraction with a 24:1 mixture of chloroform and isoamyl alcohol is done in which chloroform helps to remove slight traces of phenol contamination from the nucleic acid. The purpose of adding isoamyl alcohol is to prevent foam formation.

All the 47 isolates were confirmed as *Phytophthora colocasiae* by yielding a positive amplification with the previously reported *P. colocasiae*- specific primers. Based on the rDNA ITS region, Misra *et al.* (2010) had reported a conventional PCR assay for the *P. colocasiae* isolates. Unfortunately, the preliminary tests of this assay was a failure. When DNA from recent *P. colocasiae* isolates used in this assay, several problems arise in the amplification process regarding its specificity. Reports of Attallah *et al.* (2007) clearly states that rDNA copy number changes with the growth stage and age of the organism. So it is estimated that this could affect the accuracy of identification and proper quantification of the isolates.

Genetic diversity from fine spatial scale were analysed using ITS amplification and sequencing. The isolates were confirmed as *P. colocasiae* using ITS characterization and sequencing. Sequence alignment of all the isolates studied depicted a considerable variation in the ITS1 region. More polymorphisms were observed earlier by Cooke and Duncan (1997), in the ITS1 region of *Phytophthora* species. Considerable amount of SNPs was found among the 5 isolates collected from single taro leaf. All the five isolates included in this study were found to be different with single nucleotide polymorphisms. A similar data was presented earlier by Nath *et al.* (2013). They reported that no isolate from single leaf was clustered together as they are genetically different from each other The possible reasons that could be attributed to the diversity of pathogen include rapid asexual reproduction by the synthesis of numerous sporangia, which either directly germinate or differentiate into motile zoospores. Mutation can be considered as another vital source for genetic variability in oomycete pathogens as per the reports of Goodwin (1997).

5.3 SSR analysis of *Phytophthora colocasiae* isolates

In the present study, reported 10 SSR markers (Schena *et al.*, 2008) were utilized for the diversity analysis of 47 *P. colocasiae* isolates isolated from diverse geographical locations of India and grouped into 5 distinct populations based on its geographical origin. Schena *et al.* (2008) developed SSR primers for wide range of *Phytophthora* species using unigene datasets. These were available publically and get extracted from genome sequence data of *P. sojae, P. infestans* and *P. ramorum. The two approaches they employed were* identification of polymorphic SSR loci common to many *Phytophthora* species which yielded 171 reliable sequences containing 211 SSRs and a second approach of useful loci identification which are common to some *Phytophthora* species more closely related to *P. sojae* like *P. europaea, P. cambivora, P. fragariae and P. alni.* Identification of SSR containing loci and creating a microsatellite marker pool for *Phytophthora colocasiae* populations were defined as the prime goal of their study. Before doing the analysis, screening

of 25 different SSR primer combinations developed by Schena *et al.* (2008) was performed. Primers which produced two or more bands were selected for the study and their annealing temperature was determined using gradient PCR. Finally, we selected only ten out of the total primers as they gave considerable clear and good bands.

Schena *et al.* (2008) selected 55-58 °C as annealing temperature for the SSR analysis. But in gradient PCR, we get an annealing temperatures ranging from 53.5°C-57.4°C. A generally accepted rule is that the annealing temperature should be at least 5°C less than that of the melting temperature (Yeh *et al.*, 1999). But according to Schena *et al.* (2008), the value is not optimal most of the times and should determine annealing temperature manually via gradient PCR. After resolving the amplicons using selected SSR primers under the determined annealing temperatures, clear polymorphic bands were obtained for all the primers selected. All the primers used in this study gave 100% polymorphism.

Lees *et al.* (2006) developed SSR markers for *Phytophthora infestans* by screening EST and BAC sequences for SSR motifs. They reported that on testing the primer pairs with 10 isolates of *P. infestans*, nearly 10% were found to be polymorphic in nature and further, one additional polymorphic marker was constructed by partial genomic library screening. All loci were amplified successfully with majority of them found to be polymorphic, when other *Phytophthora* species were genotyped They also stated the possibility of transferring the present study to closely related taxa.

The studies by Cissin *et al.* (2015) showed that SSRs developed from *P. sojae*, *P. ramorum* and *P. infestans* genome could easily be transferred to *P. capsici*. Earlier the same primer sets were tested on 16 different species of *Phytophthora* and successful cross species amplification could be elucidated (Schena *et al.* 2008). In our study, out of 25 genomic SSRs ten had successful PCR amplifications with *P. colocasiae* genomic DNA. Schena *et al.* (2008) reported that successful cross species amplification could be obtained with (25%) primers from *P. sojae*, 33.33% from *P. infestans* and 71.42% from *P. ramorum*. Among the 10 genomic SSR

primers, six primers amplified more than five fragments. Cissin *et al.* (2015) reported that be due to the presence of multiple priming sites in the genome, more than one loci could be amplified with most of the primers used. All the 10 primers generated polymorphic bands among the isolates tested in this study. The number of alleles was found to be in the range of 2 to 9 with an average 5 alleles per locus. The highest average PIC value of 0.819 was exhibited by primer combination I9F/I10R. The primer combination S12F/S13R showed least value for PIC (0.359). The higher H_E value of I9F/I10R primer combination indicate the highest level of diversity exhibited by that molecular marker.

5.4 Analysis of molecular marker data

Pathogens having elevated genetic diversity levels, enormous population size, a mixed reproductive system, and higher rates of mutation often shows an increased evolutionary potential (McDonald and Linde, 2002). So information regarding evolutionary potential and population size of *P. colocasiae* is very essential for making disease management strategies to reduce leaf blight disease incidence. For implementing suitable breeding programs, knowledge regarding the evolutionary potential of pathogen populations is highly important. SSR marker based diversity analysis can expose polymorphisms among highly related isolates from the same field. These variations can be due to the greater selection pressures inflicted by fungicides and host resistance on the pathogen. Earlier SSR markers have been used for assessing the genetic variability and population structure of other *Phytophthora* species (Zhu *et al.*, 2004; Lees *et al.*, 2006; Chmielarz *et al.*, 2014).

Using the molecular scoring data, Hierarchical clustering was done and the 47 *P. colocasiae* isolates were divided into two major clusters in which the 2nd cluster was having the maximum number of isolates. While analyzing the obtained cluster result with that of geographical regions, 7 isolates from Odisha with cottony morphology in PDA were grouped together in the first cluster. All the isolates collected at fine spatial scale were grouped together with highest similarity.

Likewise isolates from Kerala and Andhra Pradesh found to be clustered together as there exist less geographical distance between these two places.

An intense genetic variation between the isolates were observed, when genetic distance was estimated through SSR analysis. In the *P. colocasiae* *populations, migration events are common and this results in sharing of a common clade by isolates from different fields.* Isozyme and RAPD markers based diversity study failed to group the *P. colocasiae* populations based on their geographical origin (Lebot *et al.*, 2003; Mishra *et al.*, 2010). No correlation was found between molecular marker data and geographical origin of pathogens in the earlier studies (Schilling *et al.*, 1996; Day *et al.*, 2004; Linzer *et al.*, 2009; Cardenas *et al.*, 2011). Isolates obtained from the same geographical area exhibited a diverse SSR patterns and were separately clustered. Like fungi, *P. colocasiae* isolates were capable of recombination and they are highly clonal (Taylor *et al.*, 2000). The SSR diversity in *P. colocasiae* isolates suggests that genetic recombination is possible in this pathogenic oomycete.

AMOVA analysis of 47 *P. colocasiae* isolates indicated that variation within the population is 34%. The highest percentage of variation was found among the populations. These results depicted that there exists higher variation between the five different populations. Moreover, the pair- wise Phi PT values between two populations depicts higher genetic differentiation between isolates collected at fine spatial scale and isolates from Odisha. Genetic differentiation was found to be negligible between isolates from Kerala and isolates collected at fine spatial scale. In PCoA distribution of five different populations is shown in a significant way and the first coordinate axis accounted for 64.51% variation. In PCoA all the isolates from Odisha region were clustered together. In factorial analysis there exist close relationship between isolates from Odisha and Assam. A close agreement was observed between the results arising from and factorial analysis, results of STRUCTURE analysis, PCoA and clustering. In STRUCTURE analysis isolates from Odisha and Assam were grouped together. The subpopulation 2 of

STRUCTURE was found primarily in quadrants II and III of factorial analysis. The structure harvester calculated best *K* value at 2.

The calculated values of *G*ST and *N*m between populations showed that a low level of *G*ST was due to a higher *N*m value greater than the genetic differentiation threshold of 1 according to Wright (1978). The estimated gene flow (*Nm*) was found to be 0.402. This results indicate that the genetic differentiation among populations of the entire distribution area is significant and that gene flow is limited. Studies by Garant *et al.* (2007) has shown earlier that *N*m plays a vital role in genetic differentiation, species diversity and also has a positive impact on the adaptive evolution of species. Gene exchange was perhaps caused by migration of the oomycetes along with corms or by transmission of the oospores, which was appropriate for explaining the close relationship between isolates separated by a large geographic distance. Isolates collected at fine spatial scale showed negligible diversity between them as the value of He and I found to be zero.

The reason behind the high levels of genetic variation in populations of *P. colocasiae* isolates is not very clear. But it is evident that sexual recombination can increase the chances of genotype diversity in populations, as it produces unique recombinants. The management of leaf blight has become highly challenging with the introduction of new genotypes in sexually reproducing populations. This causes increased variability in pathogen features like resistance to normally used fungicides and higher degree of aggressiveness. However, there exist rare sexual reproduction events in *P. colocasiae* as it is a heterothallic species that requires both its mating types (A1 and A2); which is rarely occur in the same field (Lin and Ko, 2008; Mishra *et al.*, 2010). It is believed that the genetic diversity of *P. colocasiae* populations can be due to events like mitotic recombination. As per the reports of Goodwin, (1997) genetic variation in *P. colocasiae* can be due to events like chromosomal deletions, duplications, translocation and mutation. Higher frequencies of mitotic gene conversion in *Phytophthora sojae* contributes to its variability (Chamnanpunt *et al.*, 2001).

There are reports regarding *P. infestans* asexual progenies in terms of several characters like colony morphology, virulence degree and growth rate get diverged from their parents (Caten and Jinks, 1968; Abu-El Samen *et al.*, 2003). In general, we can state that populations having higher isolate sizes tend to exhibit more diversity, as through mutation new alleles were raised and certain alleles were lost due to random genetic drift (Hartl and Clark, 1997). Even though this study cannot provide information or reasons on the effective population size and information regarding gene flow, it still provides important evidence on the evolutionary potential of the Indian *P. colocasiae* populations.

SUMMARY

6. SUMMARY

The study entitled "Genetic diversity analysis of *Phytophthora colocasiae* using SSR markers" was carried out at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2016-2017. The objective of this study was to analyse the genetic variation in *Phytophthora colocasiae* populations using SSR markers. Thirty- seven isolates of *P. colocasiae* from all over the country along with ten isolates collected from ICAR- CTCRI taro fields at fine spatial scale were selected for the characterization.

The study was divided into two phases – culture characterization and genetic diversity analysis using SSR markers. The culture characterization started with the comparative evaluation of growth rate and sporangial production of *P. colocasiae* isolates on 6 different media. The recorded data was analyzed using various statistical tools such as ANOVA and DMRT test. Among the 6 different culture media tested, carrot agar was found to be the best media for growth and sporangial production of *P. colocasiae*. Based on the colony appearance of *P. colocasiae* in PDA medium, they were classified in to 8 different morphotypes. A pathogenicity assay was performed and the degree of virulence produced by each isolates was tested using a floating leaf disc method of pathogenicity assay. After the morphological and pathological analysis, DNA was extracted using three methods –Genomic DNA purification kit, using Cooke and Duncan (1997) method and by modifying the Cooke and Duncan method. Good quality DNA ranging from 1.82 – 1.88 was obtained while using modified Cooke and Duncan method and the extracted DNA was resolved in 1% agarose gel. All the 47 *P. colocasiae* isolates included in this study were confirmed at molecular level by PCR amplification using species specific primers (Nath *et al.*, 2014). ITS characterization and sequencing was performed by amplifying the rDNA ITS region of 5 *P. colocasiae* isolates collected at fine spatial scale. After aligning the sequences using CLUSTAL W (Thompson *et al.*, 1994), considerable amount of SNPs were found. For the genetic diversity analysis of 47 *P. colocasiae* isolates, a total of 25 reported

SSR primers (Schena *et al.*, 2008) were taken for the screening process. 10SSR primers were finally selected and their annealing temperatures were determined by gradient PCR. The selected primers used an annealing temperature of 53.5- 57.4 ^{0}C. After the final PCR using the primers, the product was resolved in 2% agarose. The primers showed 100% polymorphism and the number of bands ranged from 2 to 9. The genetic differentiation among the 5 populations based on geographical location of *P. colocasiae* was significantly higher (Fst=0.462, P<0.001). The results of the frequency based Fst analysis revealed that the genetic differentiation among populations throughout the entire distribution area is significant and that gene flow is restricted. The genetic diversity of each population was calculated by grouping the 47 isolates in to five populations based on their geographical locations. Observed number of alleles (N_A), effective number of alleles (N_E), Nei's gene diversity (H) and Shannon's information index (I) were calculated. H and I were found to be 0 for the population containing isolates collected at fine spatial scale. While calculating the significant deviations from Hardy–Weinberg equilibrium (HWE) at each locus, all the 10 SSR loci were found to be significantly deviated from HWE (P<0.001). Using the molecular scoring data, Hierarchical clustering was done and the whole 47 isolates were divided into two clusters in which the 2nd cluster was having the maximum number of accessions. Isolates collected using fine spatial scale were clustered together. Seven isolates collected from Odisha were grouped in cluster I. AMOVA based on Phi PT values was performed and the results indicated a 66% variation among the populations and 34% variation within the populations. The pair- wise Phi PT values of two populations were compared and estimated the genetic distances between the populations. The highest genetic differentiation was observed between population containing isolates of Odisha and population of isolates collected at fine spatial scale. Principle coordinate analysis (PCoA)was done using the same molecular data, and it is observed that isolates from Kerala and isolates from Andhra Pradesh were grouped together in the same coordinate. The result of factorial analysis was found to be in close agreement with the result of STRUCTURE analysis. The results of STRUCTURE analysis grouped the whole isolates into two populations, as we got maximum value of ΔK at K=2.

While performing the morphological, pathological and molecular characterization of *P. colocasiae* isolates collected from different geographical locations of India, it is observed that isolates from Odisha with a characteristic cottony morphology on PDA medium showed comparatively higher degree of virulence on pathogenicity test. These isolates were found to withstand higher temperature (above 28^0C) and showed higher growth rate in PDA medium. Moreover, the results of molecular level diversity study indicate that isolates from Odisha were found to be highly diverged from other isolates. The isolates collected at fine spatial scale exhibited negligible genetic diversity with each other.

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7. REFERENCES

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APPENDICES

APPENDIX I

CULTURE MEDIA- COMPOSITIONS

Potato dextrose agar medium (PDA)

Potato	250 g
Dextrose	20 g
Agar	20 g

Carrot agar medium (CA) pH 7.0

Carrot	250 g
Agar	20 g

V8 agar medium pH 7.0

V-8 juice	50 g
CaCO3	0.2 g
Agar	20 g

Czapek dox Dextrose Agar

Sucrose	30 g
Sodium citrate	2 g
Dipotassium phosphate	1 g
Magnesium sulphate	0.50 g
Potassium chloride	0.50 g

Carrot potato agar pH 7.0

Carrot	125 g
Potato	125 g
Agar	20 g

Yeast Peptone Glycerol agar

Yeas extract	10 g
Peptone	20 g
Glycerol	20 mL
Agar	20 g

APPENDIX II

REAGENTS FOR DNA EXTRACTION

SDS Extraction Buffer

Tris - HCL	1M	
EDTA (pH=8)	0.5 M	
NaCl	5 M	
SDS	1%	
β-mercaptoethanol	0.2% (v/v)	Added freshly prior to grinding
PVP	1% (w/v)	
Distilled water		

TE BUFFER (10X)

Tris – HCl (pH 8.0)	10 mM
EDTA	1 mM

TBE Buffer (10 X)

Tris base	107g
Boric acid	55g
0.5 M EDTA (pH 8.0)	40ml

Final volume made up to 1000ml with distilled water and autoclave before use.

TAE Buffer (50 X)

Tris-base	242 g
Glacial acetic acid	57.1 ml
0.5M EDTA	100 ml

Dissolve in 600 ml of distilled water, adjust the pH to 8.0 and make up to 1L with distilled water.

Ethidium Bromide (10 mg ml^{-1})

Add 1 g of Ethidium bromide to 100 ml of water. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in Aluminium foil or transfer the solution to a dark bottle and store at room temperature.

Gel loading dye (6X)

Bromophenol blue (w/v)	0.25%
Xylene cyanol FF (w/v)	0.25%
Glycerol	50%
EDTA (pH 8.0)	10 mM

Dissolve these components in nuclease free water and store at -20 ^{0}C.

APPENDIX III

ANTIBIOTIC STOCKS

Ampicillin stock solution (100 mg ml^{-1})

Dissolve 5 g of ampicillin sodium salt in 50 ml of deionized water. Filter-sterilize and store in aliquots at -20°C.

APPENDIX IV

NCBI SUBMISSIONS

KY432685.1 *Phytophthora colocasiae* isolate CTCRI PC 5-16 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

KY432684.1 *Phytophthora colocasiae* isolate CTCRI PC 4-16 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

KY432683.1 *Phytophthora colocasiae* isolate CTCRI PC 3-16 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

KY432682.1 *Phytophthora colocasiae* isolate CTCRI PC 2-16 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

KY432681.1 *Phytophthora colocasiae* isolate CTCRI PC 1-16 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

ABSTRACT

"GENETIC DIVERSITY ANALYSIS OF *Phytophthora colocasiae* USING SSR MARKERS"

By

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ABSTRACT

The main objective of this study was to analyze the genetic variation in *Phytophthora colocasiae* populations using SSR markers. This knowledge can be further exploited for the development of a good breeding strategy and utilization of plant resistance mechanisms for the effective disease management.

Morphological characterization of 37 *P. colocasiae* from all over the country and ten isolates collected from ICAR- CTCRI taro fields at fine spatial scale were performed on 6 different media. Based on the colony appearance in PDA medium, they were classified in to 8 different morphotypes. Genomic DNA extraction using modified Cooke and Duncan (1997) method yielded good quality DNA. The annealing temperatures of the 10 SSR primers (Schena *et al.*, 2008) were calculated through gradient PCR. The primers showed 100% polymorphism and the number of bands ranged from 2 to 9. All the 10 SSR loci were found to be significantly deviated from HWE ($P<0.001$). The genetic diversity of each population was calculated by grouping the 47 isolates in to five populations based on their geographical locations. Nei's gene diversity and Shannon's information index were found to be 0 for the fine spatial scale isolates. Using the molecular scoring data, Hierarchical clustering was done and the 47 isolates were divided into two clusters in which the 2nd cluster contain maximum number of isolates.

While performing morphological, pathological and molecular characterization of *P. colocasiae* isolates collected from different geographical locations of India, isolates from Odisha with a characteristic cottony morphology on PDA medium showed comparatively higher degree of virulence in the pathogenicity test. Moreover, these isolates can withstand temperature above 30^0C. The results of genetic diversity analysis indicate that isolates from Odisha were found to be highly diverged from other isolates. The isolates collected at fine spatial scale exhibited negligible genetic diversity with each other.