Training manual
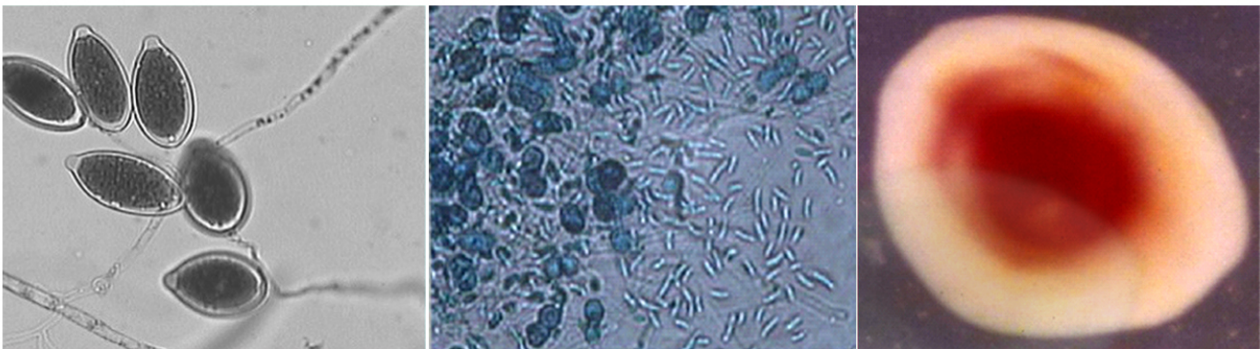
SHORT-TERM TRAINING ON

APPLICATION OF GENOMICS AND BIOINFORMATICS IN *PHYTOPHTHORA/RALSTONIA* RESEARCH

8 – 17 FEBRUARY 2011

PhytofuRa
AN ICAR OUTREACH PROJECT ON
Phytophthora, Fusarium & Ralstonia

Indian Institute of Spices Research
Indian Council of Agricultural Research iiSr
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iiSr

Short-term Training on
Application of Genomics and Bioinformatics in
Phytophthora/*Ralstonia* Research
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TRAINING MANUAL

ICAR

INDIAN INSTITUTE OF SPICES RESEARCH
(INDIAN COUNCIL OF AGRICULTURAL RESEARCH)
CALICUT – 673 012, KERALA

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PHYTOFURA – Overview

M. Anandaraj

Wilt pathogens have an enormous impact on the national economy by destroying valuable crops. Among these, the role of oomycete pathogen *Phytophthora* spp., fungal pathogen *Fusarium* spp. and bacterial wilt pathogen *Ralstonia solanacearum* are quite significant. They cause havocs to a number of horticulture and field crops of the country. Better understanding of fungal-plant interactions and pathogenicity factors is a crucial prerequisite for the development of novel disease control strategies. Recent advances in sequencing and genomic techniques have made it possible to monitor gene expression changes at the whole-genome level, which has impacted all aspects of biological sciences. Because our knowledge of molecular mechanisms of fungal pathogenesis is limited, comparative and functional genomic studies offer great promise to improve our understanding of host-pathogen interactions.

Many important plant pathogenic fungi like the ascomycetes *Fusarium graminearum,* the causal agent of Fusarium head blight (FHB) of wheat and barley and *F. verticillioides*, a causal agent of kernel and ear rot of maize, three *Phytophthora* species viz. *P. infestans*, *P. sojae* and *P. ramorum* as well as *R. solanacearum* have been completely sequenced. Comparative genomics is a powerful approach to address evolutionary and phylogenetic questions. In closely related plant pathogenic fungi/bacteria, comparative analysis can be used to improve de novo gene prediction and identify genes involved in host range determination, infection-related morphogenesis, and virulence.

One challenging task for the research community will be to apply the rich information gained in genomic studies to improve crop production and agricultural practices. As genomics and proteomics studies are not cost-effective, the research community has to combine resources by promoting collaborations within the community as well as between industry and academia. To facilitate and encourage this, Indian Council of Agricultural Research (ICAR) has launched an Outreach Project on *Phytophthora*, *Fusarium* and *Ralstonia* Diseases of Horticultural and Field Crops in the year 2008. The project envisages to study the diversity of these pathogens and host-pathogen-microbial interactions, aims to develop diagnostic and detection methodology for each of them and to develop management strategies against them. The project is operational in 17 centres distributed in nine states. Infrastructure build up and human resource development are also planned through this project. The six thematic areas are: Biodiversity, diagnostics, epidemiology, genomics, host resistance, disease management and HRD.

PHYTOPHTHORA

Plant pathogenic oomycetes cause diseases on all types of plants, in all sorts of environments, around the world. There are over 83 species of *Phytophthora* that are particularly destructive. They exhibit morphological features analogous to pathogens in Kingdom Fungi, but reside in Kingdom Stramenopila with diatoms and brown algae. They are currently classified in the Peronosporomycetes within the Oomycota, although phylogenetic relationships within this group remain in question. *Phytophthora* has a wide host range and the damage caused by *Phytophthora* on crops like potato, black pepper, cardamom, coconut, arecanut, cocoa, citrus, vegetable crops like chillies, capsicum, tomato, fruit crops such as papaya, guava, passion fruit etc. result in economically significant yield losses. *Phytophthora* can infect all parts of a plant, including the roots, stems, leaves and fruit at any stage of growth, and can be seed borne, surviving in the soil and on host debris for months. But infection most commonly occurs during periods of heavy rainfall and high humidity in plantings that are over-crowded, over-fertilized with nitrogen or where poor drainage or excessive irrigation occurs.

Phytophthora diseases are difficult to control in the tropics because of its wide host range and environmental conditions that are conducive to disease development. Generally the infection goes unnoticed until symptoms like foliar yellowing or wilting appears.

The genomes of three *Phytophthora* were recently completely sequenced resulting in novel insights on the evolution and pathogenesis of Oomycetes. Whole-genome sequencing of oomycete species began with *Phytophthora sojae* (soybean root rot pathogen; 95 Mb genome) and *Phytophthora ramorum* (sudden oak death pathogen; 65 Mb genome) because they have smaller and simpler genomes than the 240 Mb *P. infestans*. There remains a high degree of conserved synteny among all three genomes, and a core set of some 9,500 orthologous genes is present in each of the species. The total number of genes annotated for each genome is similar, ranging from 14,451 (*P. ramorum*) to 16,988 (*P. sojae*) and 17,797 (*P. infestans*). The application of molecular and DNA sequencing analyses has altered our view of oomycetes and provided new insight into the evolution of these organisms and how they have excelled as plant pathogens.

FUSARIUM

The genus *Fusarium* collectively represents the most important group of fungal plant pathogens, causing various diseases on nearly every economically important plant species. Wilt diseases by *Fusarium* spp. is one of the most serious disease problems of several agricultural, vegetable and fruit crops. *Fusarium* is a large genus of filamentous fungus widely distributed in soil, plants, animals, arthropods and humans. Though most species are harmless saprobes and are relatively abundant members of the soil microbial community, a few of them, particularly the members of *F. oxysporum* Schlecht. Emend. Snyder and Hansen are responsible for devastating vascular wilt disease in plants of over 100 cultivated plant species, including important crops such as tomato, potato, sugarcane, bean, cowpea, date and oil palm, as well as cooking and dessert bananas. The host range of these fungi is extremely broad, including a range of both gymnosperms and angiosperms. While collectively, plant pathogenic *F. oxysporum* strains have a broad host range; individual isolates usually cause disease only on a narrow range of plant species which has led to the idea of "special form" or forma speciales in *F. oxysporum*. New races of the pathogen continue to evolve, overcoming deployed resistance and thwarting plant breeding efforts. Of equal concern is the health hazard posed to humans and livestock by the plethora of *Fusarium* mycotoxins. The genus is known to secrete fumonisin mycotoxins in maize and trichothecene & estrogenic mycotoxins in cereals (*F. verticilloides* and *F.graminearum*).

Besides their economic importance, species of *Fusarium* also serve as key model organisms for biological and evolutionary research. So far genome sequences (both chromosomal and mitochondrial) of *F. graminearum*, *F. verticilloides* and *F. oxysporum* have been published. Comparative genome analysis revealed that the number of genes is more in *F. oxysporum* than other two species besides its larger genome size.

Isolation, Culturing and identification of *Phytophthora*

R. Suseela Bhai and K.B. Vinitha

Domain:	Eukaryota
Kingdom:	Chromalveolata (Stramenopila)
Phylum:	Heterokontophyta
Class:	Oomycetes
Order:	Peronosporales
Family:	Pythiaceae
Genus:	*Phytophthora*

Phytophthora spp. is mostly pathogens of dicotyledons, and is relatively host-specific parasites and is of considerable economic importance. *Phytophthora* is sometimes referred to as a fungal-like organism but it is classified under kingdom: Chromalveolata (formerly Stramenopila and previously Chromista). This is a good example of convergent evolution. *Phytophthora* is morphologically very similar to true fungi and are more closely related to plants than animals. When fungal cell walls are made primarily of chitin, Stramenopiles cell walls are made of cellulose.

Isolation of *Phytophthora* from soil
For any disease survey to be effective it is essential that both the recovery and detection methods be efficient and practical. In surveys involving *Phytophthora* spp., which because of their slow growth are rapidly overgrown by other fungi in culture, conventional isolation methods are normally ineffective.

The poor recovery of *Phytophthora* spp. from the soil may be because these fungi normally give rise to fewer infective propagules per gram of soil than most other soil fungi. Moreover because of their slow growth, it cannot compete with certain other fungi in non-sterile soil.

Depending upon the objective of the studies, soil samples are to be collected from different depths such as (0-8cm) (surface layer)   8-15cm and 15-30 cm respectively. The collected samples must be kept separately in sterilized bottles or polythene bags and examined for microbial population at the earliest (i.e., within 10-15 days). If the interval is longer, the variations in population will be more.

A number of techniques have been designed for the selective isolation of *Phytophthora* from the soil such as soil baiting using suitable baits, soil dilution plating, soil sieving technique etc. Selective media have been devised for the isolation of *Phytophthora* from the soil which includes different antibiotics and selective antifungal compounds (Appendix-1)

Baiting
Plant material can be used as bait to isolate *Phytophthora* from the soil. Suitable baits include stem, leaves, surface sterilized roots, fruits etc. Baiting techniques are appropriate for zoosporic fungi as the zoospores produced from sporangia can actively move towards and infect the bait. The suitable baits can be applied to soil suspensions or to water in which pieces of diseased roots or leaves have been added in order to trap these fungi. *Phytophthora capsici* can be isolated using leaves of Albizzia (Anandaraj *et al*., 1990) as bait, whereas *P. meadii* can be isolated using *Bauhinia variegata (*Suseela bhai1998*)* leaf discs as baits.

Materials required

Beaker (100ml) or polythene cup, distilled water and glass rod.

Procedure

- ❖ Collect soil along with roots from the rhizosphere of the infected plant
- ❖ Sieve the test soil to < 2 mm fractions.
- ❖ Put 25g of the sieved soil into the beaker or polythene container and add 100 ml distilled water.
- ❖ Mix the contents thoroughly using a glass rod to make a soil-water suspension.
- ❖ Float 10-20 leaf lets of *Albizzia falcata* (for *P. capsici), Bauhinia variegata* (for *P. meadii/ P. nicotianae/ P. capsici*) or any other suitable baits for other *Phytophthora* species with the adaxial surface in contact with water.
- ❖ Cover the beaker with thin polythene sheet to retain humidity.
- ❖ Incubate under laboratory conditions at 20-24°C.
- ❖ Daily observe for infection of leaves. Usually infection starts with in 24hrs. In case of *P. capsici* or *P. meadii*, infection occurs within 72h.
- ❖ Remove the infected leaves at exactly 72h and place it on a glass slide and observe for coenocytic mycelia growth with typical sporangia coming out from the edge of the leaf let/disc under the microscope (After 72hr, the baits will be infected by other fungi which will give erroneous results).
- ❖ Surface sterilize the infected baits with 0.1% sodium hypochlorite for 1 min.
- ❖ Rinse with three changes of sterile water, blot dry on sterile filter paper and plate it on selective medium.

Direct isolation from the soil

a) Direct plating technique:
In this method finely ground soil is taken and is spread over plates containing selective media. The plates are incubated for in dark 48-72h under laboratory conditions.

b) Soil sieving technique (P. cinnamomi)

The method used is similar to that described by McCain *et al.* (1967). This involves suspending 50 g of soil in 500 ml distilled water and agitating in a blender at a slow speed for one minute. Before the suspension settles, it is passed through a nest of sieves with aperture sizes of 149, 61, 44 and 38 u. All material remaining in the blender is washed three times and the suspension decanted through the nest of sieves. Immediately after this, several 1 g samples of soil from the 38 μ aperture sieve are plated on to selective medium and incubated at 25 °C for 24 to 48 hours before the colonies are counted.

Quantification of Phytophthora in the soil

The inoculum load of *Phytophthora* present in the soil / required for causing the disease is called "Disease Potential Index" of the soil. This can be quantified using either by soil dilution end point method or by direct plating of measured quantity of soil in selective media. However, the most common and appropriate method is the soil dilution end point method as described by Tsao(1960).

a) Soil dilution end point method:

25g soil sample was serially diluted with sterile soil viz. 1, 2, 4, 8. 16, 32, 64, 128, 256 etc and baited as above in 100ml of deionised distilled water. Count the no. of leaf disc/bait

infected and the reciprocal of the smallest fraction from which *Phytophthora* could be isolated is taken as the DPI of the soil.

b) Soil dilution plate technique:

Materials required:
Sterile Petri plates, sterile water blanks of 9ml (in vials) and 99ml (in 250ml conical flasks.), pipettes (1ml), sieve of 2mm mesh size and selective medium.

Procedure
 ❖ Collect soil from the base of the plant or from the rhizosphere.
 ❖ Air dry the soil by spreading on a sheet of paper.
 ❖ Sieve the soil using 2 mm sieve.
 ❖ Weigh 10g soil and disperse in 90ml sterile water taken in 250ml Erlenmeyer flask
 ❖ Shake the flask thoroughly to disperse the fungal propagules adhering on the surface of the soil particles.
 ❖ Pipette out 1ml of this suspension to 9ml sterile distilled water in vials (Increase the no. of dilution depending on the spore load).
 ❖ Pipette out 1ml of the above suspension into sterile Petri dishes and pour 15ml of selective media (of bearable warm) and rotate the plate to dispense the soil solution.

Incubate the plates at 20-24°C for 72h. Sub culture the colonies formed into Carrot Agar, Corn meal agar or Water agar slants and maintain the colonies in BOD incubator at 15°C for further use.

Isolation of *Phytophthora* from aerial plant parts
Isolation of *Phytophthora* from plant tissues can be done by placing small portions of infected tissue taken from the advancing edge of lesions on to a suitable growth medium in sterile Petri dishes under aseptic conditions. Surface sterilization of the excised tissue before plating is necessary to avoid surface contamination by saprophytic organisms that commonly grow over plant surfaces.

For all isolations, infected portion is to be taken from the leading edge of the lesion. Ingredients such as fungicides and antibiotics in the selective media may sometimes suppress the outgrowing of hyphae from plant tissue and/or the development of *Phytophthora* propagules. In such cases, isolation from plant material should be undertaken on plain agar (tap water agar) to favour the growth of the pathogen which uses the plant material as a food base, rather than the growth of saprophytic fungi.

Materials required

Tap water agar or PVPH medium Petri plates, razor blade, surface sterilants such as Sodium hypochlorite (NaOCl) solution, sterile blotting paper, sterile vials, sterile distilled water, needles, forceps, spatula etc.

Procedure

 ❖ Collect leaves / stem showing symptoms /lesions of particular diseases.
 ❖ Wash the specimens thoroughly under tap water.
 ❖ Excise small pieces of the diseases tissue from the lesion margin.
 ❖ Surface sterilize the tissues for 1-3 min in 0.1% NaOCl or in 75% ethanol for 10 minutes and then wash in three changes of sterile distilled water.
 ❖ Blot dry on a sterile filter paper.

❖ Place on to previously poured agar in Petri plate and incubate at 24-28°C (For selective isolations, specific media should be used).

In the case of stem lesions (when there are internal lesions of the vascular tissues, careful excision may avoid the need for surface sterilization), the sample is split longitudinally from healthy to diseased area using a clean blade and small slivers of tissue from the edges of the lesions are plated out using sterile forceps. In case of capsule rot of cardamom, pure culture of *P. meadii* can be isolated by plating the surf ace sterilized seeds from the infected capsules.

Isolation of *Phytophthora* from roots
Wash the root system thoroughly under running tap water to remove the soil particles adhering to it. Excise out approximately 1cm length of roots showing the symptoms. Surface sterilize as described above and wash in three changes of sterile water. Blot dry on a sterile filter paper and place it on to previously poured agar in sterile Petri dishes.

The presence of phenolic compounds makes the isolation of *Phytophthora* difficult. So it is necessary to reduce the amount of phenolics in the plant part (Eg. Black pepper). Leaching method is found to be helpful in this case.

➢ Wash the infected roots thoroughly with tap water to remove the soil particles from it.
➢ Take a clean muslin cloth and wrap the root sample in it.
➢ Tie it on a tap and allow water to flow through it for overnight.
➢ Take out the sample.
➢ Surface sterilize as described above and wash in three changes of sterile water.
➢ Blot it dry on a sterile filter paper.
➢ Transfer it to selective medium for isolation of *Phytophthora.*
➢ Incubate it at room temperature for 72-96 h.

Purification & Culturing

Purification generally involves the isolation of desired single spore colonies from a mixture. This is also possible by making a suspension of zoospores in sterile water and streaking on agar as follows.

❖ Cut out mycelial discs from the margins of the colony and transfer it to a sterile Petri dish.
❖ Add 15ml sterile distilled water to it.
❖ Keep the plates for sporangia formation under intense light.
❖ When sporangia form, induce zoospore differentiation by placing the Petri plate in a refrigerator for 10 minutes. Bring back to room temperature.
❖ Collect the zoospores with a micropipette and place on a selective medium.
❖ Incubate the plates at 24-28°C.
❖ The single spore colonies formed may be sub cultured to agar (PDA/CMA/CA) slopes and maintain in the BOD at 15^oC for further studies.

Preservation & Maintenance

There are several methods of maintaining a culture collection of *Phytopthora*, and all aim to retain them in the condition in which they were at the time of isolation. Preservation technique range from continuous growth through methods that reduce rates of metabolism to the ideal situation where metabolism is suspended.

There are many methods available for the preservation and storage of *Phytophthora*. Continuous growth techniques involve frequent transfer from depleted medium to fresh medium providing optimum growth conditions and there are also methods that delay the need to subculture. This method involve storage on growth medium in the refrigerator, freezer (-10°C to –20°C), under a layer of oil or in water. An original culture should be preserved without sub culturing and a seed stock should be stored separately from the distribution stock. It is always advisable to keep a duplicate collection in another secure building or site as a reserve. After preservation, the viability, spore germination, purity and identity should be rechecked and compared with data recorded before preservation.

Preservation methods

a) Growth on agar slants

Cultures are normally grown in test tubes or vials on a nutrient medium. Many fungi can be maintained in this way for years. However, *Phytophthora* has to be sub cultured every six months to maintain the viability. If the culture is found to show a decrease in viability, then the culture can be passed through the host (re-inoculate to host plant) and re-isolate. Cultures can be stored at room temperature in a cupboard protected from dust. But they may dry out rapidly in tropical climates and must be transferred to fresh media at least every 2-3 months. Storage at 15°C in a BOD incubator can extend the transfer interval to 5-6 months from the average period of 2-3 months.

b) Preservation under mineral oil

Covering cultures on agar slants (0° to the horizontal) in 30ml universal bottles with mineral oil prevents dehydration and slow down the metabolic activity and growth through reduced oxygen tension. Mature healthy cultures are covered by 10mm of sterile mineral oil (liquid paraffin or medicinal paraffin Sp. gr. 0.830-0.890sterilized by autoclaving twice at 121°Cfor 15 min.). If the oil is deeper than 10mm the organism may not survive, whereas if the depth is less, exposed mycelium or agar on the sides of the container may allow moisture to evaporate and the culture to dry out.

Cultures can be retrieved from oil. This is done by removing a small amount of the colony on an inoculation needle, draining away the oil as much as possible and then streaking on to a suitable agar medium. Growth of this culture will be poor due to adhering oil, so sub-culturing is needed by re-isolating from the edge of the colony and transferring to fresh media.

c) Preservation in Water

Agar discs cut from the growing edge of the *Phytophthora* colony are placed in sterile distilled water in 15ml vials and the lids are tightly screwed down. They can be stored at 20-25°C. Retrieval is by removal of a disc and placing it on a suitable growth medium. Any growth during storage in water can be reduced if the spores or hyphae are removed from the surface of agar media and no medium is transferred. *Phytophthora* species can be stored in water for a period of 2-3 years without any loss of viability.

d) Cryopreservation

This is one of the methods for storage of micro organisms without changing the morphological or physiological characteristics. This can be achieved by storing at ultra low temperature of –190°C to –196°C in or above liquid nitrogen. The shelf life by this method is often considered as infinity. Freeze storage at –80°C has been successfully used for the shorter term giving around 15 years of storage.

Pathogenicity testing of the isolates

To prove Koch's postulates or to test the viability of the isolates the culture has to be inoculated to target plants either by root inoculation or by inoculating to aerial parts depending on the type of infection. For aerial infection, detached leaf assay is the convenient procedure.

Detached leaf assay

- ❖ Take out young leaves from a susceptible variety.
- ❖ Wash well with tap water and blot dry.
- ❖ Wipe the leaf with alcohol.
- ❖ Take plastic boxes spread with wet filter paper.
- ❖ Inoculate the leaves with 5mm mycelial discs cut out from the margins of a three day old culture.
- ❖ Place a bit of moistened absorbent cotton above the disc in order to prevent dehydration.
- ❖ Note down the lesion diameter at right angles at 24, 48 and 72 h.

Mating type determination of *Phytophthora*

Phytophthora species are either homothallic ie. producing oogonia and antheridia (sex organs) in the same culture or heterothallic or self sterile ie. producing sex organs only after mating with opposite type. If heterothallic, the mating type should be determined by dual culturing with known mating type as follows.

- ➢ Grow the unknown isolate and the known A1 or A2 isolate for pairing on carrot agar medium in petri plates.
- ➢ Cut out mycelial discs of 5mm size from the margins the cultures using a sterile cork borer.
- ➢ Plate it on Ribeiro's medium in a sterile 9 cm Petri dish in such a way that the known isolate and the unknown A1 or A2 isolate are kept at a distance of 30mm apart.
- ➢ Incubate the plates in dark at 25°C. Oospore production is optimal in darkness and reduced in light.

- ➢ Check for the production of oospores by viewing under a microscope periodically. The presence of oospores away from the known mating type ie. formation of sex organs with in the unknown type is the indication of the opposite mating type. The oospores produced at the place of meeting of two hyphae is hybrid and cannot be counted for mating type determination.

Identification of *Phytophthora* species

The taxonomic key prepared by Waterhouse (1963) revised by New hook et al. (1978) and stamps et al. (1990) based on morphological and physiological parameters is the accepted key for the morphological identification of *Phytophthora* species. (Erwin and Rebeiro, 1996) Basically the taxa are made into six broad groups (Table 1). Besides this grouping, certain other criteria are also used for identifying to the species level (Table 2) . Table 3 is an example to show how to identify an isolate based on the Key of Stamps *et al.,* 1990

Table 1. Grouping of *Phytophthora* species

Group	Characteristics	Species
1	Papillate sporangia, apical thickening hemispherical, at least 4mm deep, exit pore narrow(5-7µm), abundant sporangia in solid culture, caduceus, oospores always formed in host and in culture. Antheridia paragynous	*P. cactorum, P. clandestine, P. idaei,, P. iranica, P. italic. Pseudotsugae, P. tentaculata*
2	Papillate sporangia, apical thickening hemispherical, at least 4mm deep, exit pore narrow(5-7µm),Oospores are heterothallic, Antheridia are amphigynous	*P. arecae, P.palmivora, P.boehmeriae, P. botryose, P. capsici, P. citrophthora, P.heveae, P.katsurae, P.meadii, P.megakarya, P.mexicana, P. nicotianae (P. parasitica, P. palmivora var. palmivora, P. palmivora var.heterocystica.*
3	Sporangia semi papillate, apical thickening shallower, sometimes scarcely papillate, hyaline thickening is evident but less hemispherical, exit pore narrow(5-7µm) ,sporangia can be caduceus or not, oospores always formed in host or in single culture, Antheridia paragynous	*P. citricola, P.cyperi (not culturable, P.cyperibulbosi (not culturable) P.inflata, P.lepironiae, P. macrospora, P. porri, P.primulae, P. syringae*
4	Sporangia semi papillate, apical thickening shallower, sporangia usually deciduous, sporangiophores are sympodial, Oospores are heterothallic, anthredia predominantly amphigynous.	*P. colocasiae, P. eriugena, P.hybernalis, P.ilicis, P. infestans, P. macrochlamydospora, P.mirabilis, P.phaseoli*
5	Sporangia non-papillate, apical thickening scarce, exit pore wide(>12µm), sporangia non-caducous and never produced in media but in water or aqueous salt solution and proliferate internally, oospores always formed in host or in single culture, Antheridia paragynous	*P.fragariae.var.fragariae, P.fragariae.var.oryzobladis, P.fragariae.var.rubi, P. humicola, P. insolita, P.lateralis, P.leersiae, p. medicagenis, P.quinineae P.sojae, P.trifolii. P.verrucosa,*
6	Sporangia non-papillate, sporangia proliferate externally and internally, Oospores are heterothallic, Antheridia predominantly amphigynous.	*P.cajani, P. cambivora, p.cinnamomi var.cinnamomi, P. cinnamomi vr. parvispora, P.cryptogea, P.drechsleri, P.drechsleri var.cajani, P.erythroseptica var.erythroseptica, P.erythroseptica var.pisi, P.gonapodyides, P.japonica, P.melonis, P.richardiae, P.sinensis, P.undulata, P.vignae,*

Table 2. Criteria used for identification of *Phytophthora* species

1	Branching pattern of sporangiophores- unbranded, irregularly branched, simple sympodium, compound sympodium, umbellate
2	Sporangium apex- Papillate, semi papillate, non-papillate
3	Width of exit pore on sporangia
3	Abundance of sporangia in solid media
4	Caducity of sporangia- Caducous or non caducous
5	Pedicel length- short/medium / long
6	Sporangium shape- homothallic/heterothallic
7	Sporangium length-breadth ratio
8	Production of oogonia and oospre
9	Nature of antheridia- (amphigynous/paragynous/or both)
10	Antheridia- unicellular or bicelluar
11	Antheridia size and shape
12	Abundance of oospores on host tissue or in culture-
13	Type of sporangiophores- (unbranded, irregularly branched, in simple sympodium, in a compound sympodium, umbellate
14	Proliferation of sporangia
15	Hyphal swellings
16	Presence or absence of chlamydospores
17	Oogonium shape and size
18	Ornamentation of the oogonium wall
19	Diameter and thickness- Plerotic or aplerotic
20	Host specificity, etc
21	Chlamydospores- Terminal/lateral/intercalary/sessile
22	cardinal temperature

Table 3. Identification of *P. meadii McRae A2 Mating type and P. nicotianae var. nicotianae*

Group II - *P.meadii and P. nicotianae var. Nicotianae*				
	As per the Key (Stamps *et al.* 1990)		Isolates studied	
A. Sporangia	*P. meadii*	*P. nicotianae var. nicotianae*	*P. meadii McRae A2 Mating type*	*P.nicotianae var. nicotianae*
Papillae pore narrow	+	Oc	+	+
> 1 apex	+	Oc	Oc	Oc
Caducous, pedicel length	M	Oc	M	M
Av.l/b ratio >1.6		*	>1.6	>1.6
± spherical	*	*		
Ellipsoidal	*		+	+
Obpyriform	*	*		
Obturbinate		*		
Elongated neck in water		+		
Distorted shapes	*	+	+	+
Hyphal projections Oc		+/-	+	+
lateral attachment	*	*	+	+
Intercalary		+		
45-75μ	*	+	+	+
Residual globule	+		+	+
B. Sporangiophore				
Aerial on agar	*	+	+	+
Basal swellings Oc.	+		+Oc	
Intercalary swellings		+	+Oc	
C. Antheridia				
Amphigynous	*	+	+	+
±spherical	*	+	+	+
Ellipsoidal or ovoid	*		+	+
Stalk often eccentric		+		
<30 μm	*	+	+	+

30-40 µm	*	+	+	+
4O-50 µm	*			
Wall rough with age	+		+	+
D. Oospores				
formed readily in the host	+/-			
Frequent on isolation	+/-	+/-		
Often best in dual culture	+	+	+	+
<20-30µm	*	+	+	+
20- 30µm	*	+	+	+
30-50µm	*		+	+
Wall thick (rel. dia)	*	+	+	+
Markedly aplerotic	*	+	+	+
E. Chlamydospores				
Abundant		+		+
In some isolates only	+		+	
<25µm	+	+		
25-35µm	+	+	+	+
>35µm		+		
F.Hyphal swellings on agar		+	Oc	+Oc
In water round or angular		+		+
In chains or clumps		+		+
Large spherical			+	+
Spherical with radiating hyphae		Oc	+	+
G. Growth at >35°C		+Oc		+

*character present from original descriptions and figures.
+Oc character has particular importance   +/- present in some isolates
± more or less   < less than. > more than   **Oc** occasional   **M** medium
Ref. *J. plantation Crops* 33(3): 193-203

PREPARATION OF CULTURE MEDIA

1. POTATO DEXTROSE AGAR (PDA) MEDIUM (FOR MYCELIUM GROWTH)

Potato -   200g

Dextrose –   20g

Agar –   20g

Distilled water -1 liter

- ❖ Peel off the potato and cut it into 1 cm cubes.
- ❖ Boil the potato pieces in 500ml of distilled water till a glass rod can easily pierce through the pieces.
- ❖ Filter the broth through four layers of cheese cloth and dissolve the weighed dextrose in it.
- ❖ Add distilled water to bring up the volume to one liter.
- ❖ Add agar to the broth and boil till it dissolves completely.
- ❖ Transfer it to conical flasks and sterilize at 15 lb pressure for 20 minutes.

2. CARROT AGAR (CA) MEDIUM (FOR MYCELIUM GROWTH)

Carrot –   200g

Agar-   15g

Distilled water – 1 liter

- ❖ Peel off the carrots and blend it in a blender along with 500 ml distilled water.
- ❖ Filter through four layers of cheese cloth and squeeze out the juice from the residue.
- ❖ Add distilled water to bring up the volume to one liter.
- ❖ Add the weighed agar to it.
- ❖ Boil the mixture and again filter it through four layers of cheese cloth.
- ❖ Transfer it to conical flasks and sterilize at 15 lb pressure for 20 minutes.

3. RYE AGAR MEDIUM (FOR MYCELIUM GROWTH)

Preparing rye concentrate

Rye –   600g

Saccharose –   20g

Agar-   15g

- ❖ Disinfect the rye grains for 10 minutes in 1 litre 2% Sodium-hypochloride.
- ❖ Wash 4 to 5 times with distilled water using a sieve.
- ❖ Bring the rye over to a dry clean tub and add 500 ml distilled water.
- ❖ Cover the tub with Saran wrap and a dark cloth.
- ❖ Let the rye germinate at room temperature for 36-48 hours.
- ❖ Pour off the liquid and wash the grain twice with distilled water.
- ❖ Add 1 litre tap water and blend for 20 seconds.
- ❖ Pour the suspension in two 1 litre flasks.
- ❖ Incubate in water bath (or incubator) for 3 hours at 50°C.

- ❖ Filter through sieve and discard grains (save suspension will be about 1200 ml).
- ❖ Bring end volume up to 2 litre with distilled water.
- ❖ Store Rye concentrate at –20°C

20 % Rye Agar (RA)

- ❖ Take 200 ml rye concentrate.
- ❖ Add 20 g saccharose (table sugar) and 15 g agar.
- ❖ Bring end volume with distilled water to 1 litre.
- ❖ Autoclave at 15 lb pressure for 20 minutes.

4. CORN MEAL AGAR MEDIUM (FOR MYCELIUM GROWTH)

Corn meal agar (HiMedia)   17g

Distilled water   1l

- ❖ Add 17g corn meal agar powder in 1l distilled water.
- ❖ Boil till it melts completely.
- ❖ Transfer it to conical flasks and autoclave at 15 lb pressure for 20 minutes.

5. V8 JUICE AGAR MEDIUM (FOR MYCELIUM GROWTH)

V8 juice agar (HiMedia)   44.3g

Distilled water   1.0 l

- ❖ Add 44.3g V8 juice agar powder in 1l distilled water.
- ❖ Boil till it melts completely.
- ❖ Transfer it to conical flasks and autoclave at 15 lb pressure for 20 minutes.

6. RIBEIRO'S MEDIUM (FOR OOSPORE PRODUCTION)

Glucose -   4.5g

L-Asparagine –   0.1g

Potassium nitrate –   0.15g

Potassium di-hydrogen phosphate – 1.0g

Magnesium sulphate –   0.5g

Calcium chloride –   0.1g

β- sitosterol – 30 mg (dissolved in 30ml Di- chloro methane)

Deionised distilled water- 1000 ml

Dissolve the weighed chemicals in deionised distilled water and make up to one liter. Dispense 1 ml each of the microelement stock solution and ferric chloride stock solution into the medium.

- ➢ Adjust the pH to 6.2 with 6N potassium hydroxide
- ➢ Add 15g agar.

- Sterilize at 121°C for 20 minutes at 15 lb/ inch^2 pressure.
- Allow the medium to cool to bearable warm.
- Add 1 ml of the filter sterilized thiamine solution to the media.
- Shake the flask to disperse thiamine into the medium and pour into Petri plates.

(i) Microelements (stock solution)

Sodium molybdate –	41.1mg
Zinc sulphate –	87.8mg
Copper sulphate –	15.4 mg
Sodium borate –	0.5 mg

Dissolve the above microelements in 100 ml de- ionized distilled water.

(ii) Ferric chloride stock solution

EDTA –	2.6 g
Potassium hydroxide –	1.5g
Ferric chloride –	50 mg

Dissolve the above microelements in 100 ml de- ionized distilled water.

(iii) Thiamine stock solution

Thiamine –	10mg
Distilled water – 10 ml	

Dissolve thiamine in 10ml distilled water and sterilize this stock solution by Millipore filtration.

7. ANTIBIOTIC MEDIUM FOR SELECTIVE ISOLATION OF *PHYOTPHTHORA*

(i) PVPH MEDIUM (Tsao and Guy, 1977)

PVPH stock solution

Pimaricin solution	0.8ml
Vancomycin	224.5mg
PCNB (Pentachloronitrobenzene)	146.3mg
Hymexazol	55.3mg
Sterile distilled water	95ml

- Add 0.8ml of Pimaricin solution in 95ml sterile distilled water.
- Dissolve the weighed vancomycin, PCNB and hymexazol in 5ml DMSO (Dimethyl Sulfoxide) and add it to the above solution.
- Transfer it to sterile amber coloured bottles and store at 4°C

PVPH medium
- Prepare 100ml carrot agar /CMA

- ➤ Allow the medium to cool to bearable warm.
- ➤ Add 10ml of this stock solution to 100ml culture media and pour this to Petri plates.
- ➤ Store the plates in dark and use within five days of preparation.

(ii) $P_5ARP[H]$ MEDIUM *(Jeffers and Martin, 1986)*

$P_5ARP[H]$ stock solution

Pimaricin	2ml
Ampicillin	2.5g
Rifampicin	100mg (dissolved in 10ml DMSO)
PCNB	1g
Hymexazol	225mg
Sterile distilled water	90ml

- ➤ Add 2ml Pimaricin solution in 90ml sterile distilled water.
- ➤ Dissolve the weighed ampicillin, PCNB, rifampicin and hymexazol to it.
- ➤ Transfer it to sterile amber coloured bottles and store at 4°C.

$P_5ARP[H]$ medium
- ➤ Prepare 100ml carrot agar medium.
- ➤ Allow the medium to cool to bearable warm.
- ➤ Add 1ml of this stock solution to 100ml culture media and pour this to Petri dishes.
- ➤ Store the plates in dark and use within five days of preparation

Note: Activity of different antibiotics used in *Phytophthora* selective media

Pimaricin: Effective against most non-pythiaceous fungi.

Vancomycin: Effective against most gram positive bacteria, particularly Staphylococci resistant to most other antibiotics.

PCNB (Pentachloronitrobenzene): Primarily a soil fungicide.

Hymexazol: Inhibits *Pythium* spp. but not *Phytophthora.*

Ampicillin: Effective against most gram positive bacteria

Rifampicin: Effective against most gram positive bacteria

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Isolation, culturing and identification of *Ralstonia solanacearum*

R. Ramesh, T.P Prameela and Vijay Mahantesh

Isolation of *Ralstonia solanacearum* from wilted plant (Brinjal, tomato, chilli and ginger)

- Remove the wilted plant samples from the soil and bring to laboratory.
- Wash stem and root portion of the plant thoroughly under running tap water to remove adhering soil particles.
- Dry the samples on a blotting paper under shade.
- With the help of a knife, cut the stem at the region close to the root.
- Dip the cut end of the stem in small quantity of sterile distilled water in a test tube till the white colour ooze started flowing. In case of ginger, use the pseudostem for isolation.
- Collect the flowing ooze and streak a loop-ful of the ooze on the TZC agar plates (Kelman, 1954).
- Incubate the plates at 28 ± 2 °C for 36-48 h.
- Select pink centered, fluidal colonies and purify. Avoid red colored non-fluidal colonies.

TZC medium

Chemical	Quantity
Glucose	5.0 g
Peptone	10.0 g
Casamino acid	1.0 g
TZC	0.005%
Agar	18.0 g
Distilled water	1000 ml

Note: 2,3,5-triphenyl tetrazolium chloride (TZC) is heat labile and to be filter sterilized or autoclaved for 8 min separately. The stock should be stored in light-proof capped bottle under refrigeration. TZC stock solution should be added to the autoclaved, cooled medium to the final concentration 0.005%. Use 5.0 ml of 1.0% TZC stock for one litre of medium.

Isolation of *Ralstonia solanacearum* from soil

Serially dilute the soil sample in sterile distilled water and plate it on SMSA medium (Engelbrecht, 1994 and modified by Elphinstone *et al.*, 1996). Incubate the plates at 28 ± 2 °C for 36-48 h for the appearance of colonies.

SMSA medium

Chemicals	Quantity
Casamino acids	1.0 g
Bacto Peptone	10.0 g
Glycerol	5.0 ml
Agar	18.0 g
Distilled water	1 litre

Add the following antibiotics to 250 ml of melted media at a temperature of 50 °C

Antibiotics	Stock (%)	Final concentration (ppm)	Required volume for 250 ml medium
Polymyxin B sulfate (Sigma)	1.0	100	2.5 ml
Crystal violet	1.0	5	125 µl
Tetrazolium salts (Sigma)	1.0	50	1.25 ml
Bacitracin (Sigma)	1.0	25	625 µl
Penicillin (Sigma)	0.1	0.5	125 µl
Chloramphenicol (Sigma)	1.0	5	125 µl
Cycloheximide (Sigma) for the inhibition of fungal contaminants	1.0	100	2.5 ml

Preservation and storage:

Glycerol stock:

- Grow the pure *R. solanacearum* isolates in 5ml CPG broth (TZC medium without TZC) at 28 ± 2 ℃ for 24-36 h on a shaker.
- Take 300µl sterile glycerol in a cryo vial and add 700µl of 24-36 h old grown *R. solanacearum* culture.
- Mix the contents well and immediately store in deep freezer at -78°C.

Water stock:

- Take a loop-ful of 48 h old *R. solanacearum* growth from the CPG plate and mix with one ml of sterile distilled water in an airtight vial.
- Store the water stock in the room temperature.

Lyophilization:

- Take 4-5 loop-ful of 48 h old *R. solanacearum* growth from the CPG plate and mix with one ml of sterile 10% skimmed milk.
- Follow the lyophilization procedure using this suspension according to the manufacturer's instruction.
- Store the sealed lyophilized stock at 4 °C

Revival of stored cultures from glycerol stock:

- Inoculate 20µl from the glycerol stock into 5ml CPG medium
- Incubate the tubes at 28 ± 2°C for 24 h on a shaker.

Culturing:

Use CPG medium for culturing *R. solanacearum* for the purposes like storage, inoculation etc.

Identification of *R. solanacearum*

Phenotypic characters:

Irregular, fluidal colonies with pink centre appear after 36-48 h on TZC medium. The colonies show whirling spirals when observed closely.

Biochemical characters (Biovar determination):
Basal medium

Chemicals	Quantity
Ammonium dihydrogen phosphate	1.0 g
Potassium chloride	0. 2 g
Magnesium sulphate	0.2 g
Peptone	1.0 g
Bromothymol blue	0.03 g
Distilled water	1000 ml

- Adjust the final pH of the medium to 7.0-7.1 (an olivaceous green color) by drop wise addition of 40% (w/v) NaOH solution.
- Prepare 10% solutions of the carbohydrates.
- Filter sterilize the carbohydrate solutions.
- Dispense 0.9 ml of the sterilized broth into micro centrifuge tubes and add 0.1 ml of filter sterilized carbohydrate stock.
- Inoculate 20 µl of 2 h old grown R. solanacearum culture in each tube
- Incubate the tubes at 28 ± 2°C for 24-72 h

Biovar	Utilization of disaccharides			Utilization of alcohols		
	Cellobiose	Lactose	Maltose	Dulcitol	Mannitol	Sorbitol
1	-	-	-	-	-	-
2	+	+	+	-	-	-
3	+	+	+	+	+	+
4	-	-	-	+	+	+
5	+	+	+	-	+	-

Test	Biovar 2-A	Biovar 2- T
Acid from D (-) Ribose	-	+
Acid from D (+) trehalose	-	+
Utilization of L (-) tryptophan	-	+
Utilization of L (+) tartarate	-	+
Degradation of pectates	+	+++
Pathogenicity on potato at 25°C	high	Low

Identification by PCR

Isolation of genomic DNA

- Inoculate a well isolated colony into 5ml CPG broth and keep the tube on the shaker at 120 rpm at 28 ± 2 °C for 12 h.
- Take 1.5ml of the 12 h old grown bacterial culture (OD 0.5-1.0 at 600nm) in a micro centrifuge tube and centrifuge at 10,000rpm for 10 min at 4°C.
- Discard the supernatant and wash the pellet with sterile distilled water twice.
- Dry he cell pellet obtained on tissue paper, and add 675μl of genomic DNA extraction buffer [Tris HCl (100mM), EDTA (100mM), Na_2HPO_4 (100mM), NaCl (1.5M), CTAB (1%), Proteinase K (20μg), Lysozyme (100μg)].
- Keep the mixture at 37°C for 30 min with intermittent vortexing at every 10 min.
- Add 75μl of freshly prepared 20% SDS and incubate at 65°C for 2 h with intermittent vortexing.
- Centrifuge the tubes at 11,000rpm for 10 min at 4°C.
- Collect the clear supernatant in a new micro centrifuge tube; to which add equal volume of chloroform: isoamyl alcohol (24:1).
- Centrifuge the tubes at 10,000rpm for 10 min at 4°C and transfer the aqueous phase to a new micro centrifuge tube; to which add 0.6 volume of isopropanol and mix well.
- Keep the tubes at room temperature for 60 min and centrifuge at 10,000rpm for 10 min at room temperature.
- Wash the pellet twice with 70% ethanol; dry the pellet thoroughly and dissolve in 50μl of TE buffer (10mM Tris, 1mM EDTA) with RNase (20μg/ml) and store at -20°C.

Primers

Use *R. solanacearum* species complex specific primers for identification

Forward primer 759: 5'-GTCGCCGTCAACTCACTTTCC-3'

Reverse primer 760: 5'-GTCGCCGTCAGCAATGCGGAATCG-3'

PCR reaction mixture

10μl reaction mixture contained 1.0 μM of each primer, 200μM dNTPs, 1X Assay buffer, 10% DMSO, 20μg BSA, 0.5U Taq DNA polymerase and 50ng μl of the DNA template.

PCR conditions

One cycle of Initial denaturation at 94 °C for 3 min, 53 °C for 1 min 72 °C for 1 min 30 sec, followed by 30 cycles of denaturation at 94 °C for 15 sec, annealing at 60 °C for 15 sec and extension at 72 °C for 15 sec and the final extension at 72 °C for 5 min.

Reagents	Standard Concentration (A)	Required Concentration (B)	Required Volume (µl) (C)
Sterile water	1X	1X	3.44
1X Assay buffer	10X	1X	1.0
dNTPs	2 mM	200 µM	1.0
DMSO	100 %	10 %	1.0
BSA	50 µg/µl	20 µg/reaction	0.4
Primer 1(Rs 759)	10 µM	1.0 µM	1.0
Primer 2(Rs 760)	10 µM	1.0 µM	1.0
Taq polymerase	3U/µl	0.5 U	0.16
DNA Template	1 µl	50ng	1.0
Total Volume			10

Colony PCR

- Pick one well isolated colony using a sterile tooth pick and suspend the growth in 2 µl of sterile water in a PCR tube.
- Use this as DNA template.
- PCR reaction mixture and cycle conditions are as same as mentioned earlier.
- Amplification of 282 bp band indicates the *R. solanacearum*.

Pathogenicity testing

- Transplant twenty days old seedlings of brinjal and tomato into pots in glasshouse maintained at 26°C-30°C. Twenty five days old plants were used for testing the pathogenicity.
- Grow *R. solanacearum* in 20 ml CPG broth for 48h.
- Centrifuge the culture at 10,000 rpm for 8 minutes.
- Re-suspend the pellet in 20 ml of phosphate buffered saline. The population would be around 10^8 CFU ml^{-1}
- Drench 5 ml of the inoculum to the base of the seedling.
- Maintain appropriate control without pathogen inoculation.
- Observe for the occurrence of wilt.
- Wilt starts 3 days after inoculation in tomato and 7 days after inoculation in brinjal.
- Re-isolate the bacterium from the wilted plants to confirm Koch postulates.

Basic concepts in isolation of Genomic DNA from fungus

K. Nirmal Babu and Cissin Jose

Introduction

The application of molecular biology techniques to the analysis of complex genomes depends on the ability to prepare pure, high molecular- weight DNA. Fungal cells contain a thick rigid cell wall. The quantity and quality of DNA depends on the extent of rupturing cell wall and the degree of carbohydrate and protein contamination. The protocol for DNA isolation consists of two parts: a technique to lyse gently the cells and to solubilize the DNA, followed by one of several basic enzymatic or chemical methods to remove contaminating proteins, RNA and other macromolecules.

The mycelium is ground with glass powder and an extraction buffer that contains sodium dodecyl sulphate which is an anionic detergent which disrupts the lipid layers, helps to dissolve membranes and binds positive charges of chromosomal proteins (*histones*) to release the DNA into the solution. Ethylene diamine tetra acetic acid chelates divalent cations required for the activity of nucleases that are released from the cell which chews up DNA rapidly. DNA extraction is carried out with phenol:chloroform:isoamyl alcohol (25:24:1). Phenol denatures protein and dissolves denatured protein. Chloroform is also a denaturant and it stabilizes the rather unstable boundary between the aqueous phase and a pure phenol layer. Phenol: chloroform mixture reduces the amount of aqueous solution retained in the organic phase (compared to pure phenol phase), maximising the yield. Isoamyl alcohol prevents foaming of the mixture upon vortexing and aids in separation of organic phase and aqueous phase. DNA is precipitated with ethanol or isopropanol. In presence of relatively high monovalent cations, ethanol/isopropnaol induces transition in nucleic acids molecules which causes them to aggregate and precipitate from solution. Washing with 70% ethanol removes salts and small organic molecules.

Protocol

- Four days old young mycelium grown in Reibeiro's liquid medium was filtered through sterile Whatman No. 1 filter paper.
- Washed with sterile distilled water to remove traces of the medium and air dried to remove excess of moisture.
- 0.1g of mycelium was taken in a microfuge tube with 50mg of sterile sand or glass powder and 10mg PVPP.
- 750µl Extraction buffer was added to the microfuge tube and the mixture was ground.
- Centrifuged at 13,000 rpm for 5 minutes.
- After centrifugation the supernatant was taken in a sterile microfuge tube.
- 500µl of Tris phenol: chloroform: isoamyl alcohol (25:24:1) was added to it and inverted gently for 2 minutes.
- Centrifuged at 13,000 rpm for 5 minutes. The aqueous layer was transferred to sterile tube.
- Added 0.6 volume isopropanol and gently inverted. Centrifuged at 13,000rpm for 10 minutes.
- Discarded the supernatant and washed the pellet in 70% ethanol and inverted gently.
- Centrifuged at 13,000 rpm for 2 minutes.

- Air dried the pellet.
- Resuspended the pellet in 100µl molecular grade water or TE buffer (pH 8.0).
- 3µl RNase (5mg/ml) added and incubated for 30 minutes at 37°C and stored at -20°C.

Alternate Protocol 1

- 1 g of four days old fungal mycelium ground in liquid N_2 and immediately transferred to 5ml CTAB extraction buffer (2%w/v CTAB, 100mM Tris-Cl, 20mM EDTA pH 8.0, 1.4M NaCl)
- The contents mixed well and incubated at 65^0C for 30 minutes. DNA extracted with equal volume of chloroform: isoamyl alcohol (24:1) for 15 minutes on a rocking platform.
- The two phases separated by centrifugation at 12,000 rpm for 5 minutes.
- The aqueous phase is separated and the DNA precipitated with 2/3rd volume of isopropanol. Centrifuged at 12,000rpm for 5 minutes.
- The pellet is air dried and dissolved in 500µl of sterile distilled water.

Important consideration
- The oxidation products of phenol can damage nucleic acids and only redistilled phenol should be used. For complete deproteinization, extractions should be repeated until no protein precipitate remains at the aqueous/organic phase.
- In organic extraction, loss of nucleic acid at interface and into the organic phase can be minimized by back extracting the organic phase.
- Precipitation of nucleic acids at low concentration requires cooling to low temperatures to give good recovery. Precipitation of nucleic acids at high concentration is very rapid at room temperature.
- Isopropanol is less volatile than ethanol and takes longer to remove by evaporation. Some salts are less soluble in isopropanol compared to ethanol and will be precipitated along with nucleic acids. Extra washings may be necessary to eliminate these contaminating salts.

Precautions
- Wear gloves to avoid nuclease contamination from your hand.
- Phenol is toxic, highly corrosive and can cause severe burns to skin and damage clothing. Always use in a chemical fume hood.
- SDS is toxic, an irritant, and poses a risk of severe damage to the eyes. Do not breathe the dust.

Stocks & Solutions
Extraction Buffer and Medium

Extraction Bufffer	Added 10 ml 1M Tris HCl, 2.5 ml 5M NaCl, 2.5 ml 0.5M EDTA and 2.5ml 10% SDS to a sterile reagent bottle. Made up the solution to 50ml with sterile distilled water
TE Buffer	Added 10ml 1MTris HCl and 0.2ml of 0.5M EDTA. Made up the solution to 1000ml with sterile distilled water.
Rebeiros Medium	Added 4.5g glucose,0.1g L-asparagine,0.15g KNO_3, 1.0gKH_2PO_4, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$ and 30mg β-sitosterol (dissolved in 30ml CH_2Cl_2) to 900ml of distilled water. Dispensed 1 ml of microelements stock and 1ml of $FeCl_3.6H_2O$ stock to it. Adjusted the pH to 6.2 with 6N KOH

	and sterilized at 1210C for 20 minutes at 15 lb/inch2 pressure. After sterilization, added 1ml of the filter sterilized thiamine solution(1mg/ml)

Stocks

1M Tris-HCl	Dissolved 121.1 g of Tris base in 800ml of distilled water. Adjusted the pH to 7.5 by adding concentrated HCl. Allowed the solution to cool to room temperature before making final adjustments to the pH. Adjusted the volume of the solution to 1liter with water. Dispensed into aliquots and sterilized by autoclaving.
5M NaCl	Dissolved 292 g of NaCl in 800ml of water. Adjusted the volume to 1 liter with water. Dispensed into aliquots and sterilized by autoclaving. Stored at room temperature.
0.5M EDTA	Added 186.1 g of disodium $EDTA.2H_2O$ to 800ml of water. Stirred vigorously on a magnetic stirrer.Adjusted the pH to 8.0 with NaOH (approx. 20g of NaOH pellets). Dispensed into aliquots and sterilized by autoclaving. The disodium $EDTA.2H_2O$ will not go into the solution until the pH of the solution is adjusted to approx. 8.0 by the addition of NaOH.
10% SDS	Dissolved 100g of electrophoresis- grade SDS in 900ml 0f water. Heated to 68^0C and stirred with a magnetic stirrer to assist dissolution. Adjusted the pH to 7.0 by adding a few drops of conc. HCl, if necessary. Adjusted the volume to 1 liter with water. Stored at room temperature. Do not autoclave.
RNase A	Dissolved pancreatic RNase (RNase A) at a concentration of 10mg/ml in 0.01M sodium acetate (pH 5.2). Heated to 100^0C for 15 minutes. Allowed to cool slowly to room temperature. Stored at -30^0C.
Microelements stock	Dissolved 87.8mg of $ZnSO_4.7H_2O$, 41.1mg of Na_2MoO_4, 15.4mg of $CuSO_4.5H_2O$ and 0.5mg $Na_2B_4O_7$ in 100ml deionized distilled water.
$FeCl_3.6H2O$ stock	Dissolved 2.6g EDTA, 1.5g KOH and 50mg of $FeCl_3.6H_2O$ in 100ml distilled water.

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Quantification of DNA

Spectrophotometric method

DNA yield is determined by measuring the concentration of DNA in the sample by its absorbance at 260nm. Absorbance readings at 260nm should fall in between 0.1 to 1.0 to be accurate. Sample dilution should be adjusted accordingly.

Procedure

1. Dilute the DNA using water (water should be used as diluent when measuring DNA concentration since the relationship between absorbance and concentration is based on extinction coefficients calculated for nucleic acids in water)
2. Switch on the spectrophotometer.
3. Set the blank using water.
4. Take the diluted DNA sample in cuvette and place it in spectrophotometer.
5. Click the "Sample" button and note down the readings, which includes the DNA yield, A_{260}, A_{260}/A_{280}, etc.)
6. Repeat this for 3 times for getting an accurate data.

The ration of the readings at 260nm and 280nm provides an estimate of the purity of DNA with respect to contaminants that absorb UV, such as protein. For a pure DNA the A_{260}/A_{280} ratio should be in between 1.8-2.0. Since the purity is considerably influenced by pH, it is better to use 10mM Tris-HCl as the diluents.

Calculation

Concentration of DNA sample (µg/ml) = DNA yield in µg/ml X A_{260} X dilution factor

Total amount (µg) = concentration X volume of sample in milliliters

For example,

If the DNA yield is 20 µg/ml

A_{260} is 0.2, and

Dilution factor is 50 (1µl DNA+ 49 µl water)

Concentration of DNA sample = 20 X 0.2 X 50

= 200µg/ml or 200ng/µl

If you have 100 µl of the sample, then

Total amount = 200 X 100

= 20000µg or 20mg

Agarose gel electrophoresis

Introduction

Agarose gel electrophoresis is a simple and highly effective method for separating, identifying and purifying 0.5 to 25kb DNA fragments. During electrophoresis, water is electrolysed, which generates protons at the anode, and hydroxyl ions at the cathode. The cathodal end of the electrophoresis chamber then becomes basic and anodal end acidic. The use of a buffering system is therefore required when charged molecules are electrophoresed through a separation medium. Because the pH of the buffer used is basic, the phosphate backbone of DNA has a net negative charge and migrates towards the anode. The two buffers commonly used for electrophoresis are Tris-acetate with EDTA and Tris-borate with EDTA. TAE (1X) is used when DNA is to be recovered and for separation of large (>12 kb) DNA. But it has low buffering capacity and recirculation may be necessary for extended

electrophoretic run times (>6 hours). TBE (0.5X) is preferred for separation of small DNA (<1kb). It has increased buffering capacity but decreased DNA mobility.

Agarose undergoes a series of steps when it is dissolved; dispersion, hydration and melting/dissolution. -Dispersion refers to the separation of the particles by the buffer without clumping. Clumping occurs when the agarose starts to dissolve before it is completely dispersed, coating itself with a gelatinous layer which inhibits the penetration of water and keeps the powder inside from dispersing. Hydration is the surrounding of agarose particles by a solution. As the particles hydrate, they become small, highly concentrated gels. Melting temperature of a standard agarose gel is about 93^0C.

Molecules of linear, duplex DNA travel through gel matrices at a rate that is inversely proportional to the $\log_{10}$ of their molecular weight. The molecular weight of a fragment of interest can therefore be determined by comparing its mobility to the mobility of DNA standards of known molecular weight. This is the most valuable feature of electrophoresis, as it provides a reproducible and accurate means of characterizing DNA fragments by size.

Appropriate Agarose Concentrations for Separating DNA fragments of Various Sizes

Agarose (%)	Effective range of resolution of linear DNA fragments
0.5	30 to 1
0.7	12 to 0.8
1.0	10 to 0.5
1.2	7 to 0.4
1.5	3 to 0.2

Protocol

- The quality of isolated fungal genomic DNA was checked in 0.8% agarose gel.
- Melted 0.32gm of agarose in 40 ml of 1x TAE in a 150 ml flask by heating the flask in a microwave oven.
- Molten agarose was cooled to 50^0C and 2μl of EtBr (10mg/ml) was added.
- A gel is cast in a gel-casting tray with properly placed clean combs.
- Poured the agarose solution into the gel tray and allowed to gel at room temperature for 30 minutes.
- Once the gel is set, placed the gel casting tray in the electrophoresis chamber.
- Filled the chamber with running buffer until the buffer reaches 3-5mm over the surface of the gel.
- The combs were slowly removed.
- 10μl of DNA and 2μl of gel loading buffer was properly mixed and loaded the mix to each well without spillover.
- The gel was subjected to 3V/cm electricity for 1 hour in 1X TAE buffer.
- It was then visualized in Multi-image™ light cabinet (Alpha Imager 2200) and the gel images captured by Charge-Coupled Device (CCD) camera. The images are stored as JPEG extension file for analysis.
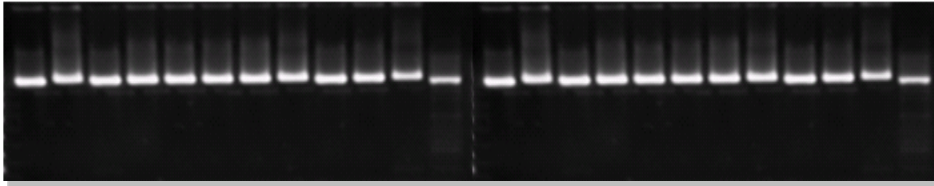
Phytophthora genomic DNA on 0.8% agarose gel

Important Considerations

- It is very important to use an adequately shielded apparatus, an appropriately grounded and regulated power supply.
- Gels are typically poured between 0.5 and 1cm thick.
- Buffer depth over the gel in a horizontal electrophoretic system should be 3-5mm. Lesser the buffer the gel may dry out during electrophoresis. Excessive buffer depth will decrease DNA mobility, promote band distortion and can cause excessive heating with the system.
- For separating large DNA molecules, it is best to run gels at both low agarose concentration and low applied voltages.

Precautions

- Ethidium bromide should be handled carefully as it is carcinogenic. Always wear gloves and goggles while handling.
- UV light is damaging to eyes and exposed skin. Protective eyewear should be worn at all times while using a UV light source.

Stocks and Solutions
Buffers

1X TAE Buffer	Added 0.8 ml of 50XTAE to 40 ml of distilled water

Stocks

50X TAE	Dissolved 242g Tris base in 800ml distilled water and added 100ml 0.5M EDTA(pH 8.0) stock solution. Added 57.1 ml of glacial acetic acid and made up the volume to 1000ml. Sterilized by autoclaving.
Ethidium Bromide	Added 1g ethidium bromide to 100ml of water. Stirred on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrapped the container in aluminium foil or transferred the solution to dark bottle and stored at room temperature.
Gel Loading Dye	Dissolved 15 mg bromophenol blue and 15mg xylene cyanol FF in 5ml distilled water. Added 100µl of 0.5M EDTA (pH8.0) and 3ml of 30 % glycerol. Made up the volume to 10 ml. Stored at 4^0C.

Decontamination of ethidium bromide solution
Method 1

- Added sufficient water to reduce the concentration of ethidium bromide to < 0.5 mg/ml.
- To the resulting solution, added 0.2 volume of fresh 5% hypophosphorous acid and 0.12 volume of fresh 0.5M sodium nitrate and mixed carefully.

Note:
- Check the pH of the solution is < 3.0
- Hypophosphorous acid is highly corrosive

Method 2
- Added sufficient water to reduce the concentration of ethidium bromide to < 0.5 mg/ml.
- To the resulting solution, added 1 volume of 0.5M $KMnO_4$ and mixed carefully.
- Added 1 volume of 2.5N HCl and mixed carefully and allowed the solution to stand at room temperature for several hours.
- Added 1 volume of 2.5N NaOH, mixed carefully and then discarded the solution.

References
Ausbel F M, Brent R, Robert E K, David DM, Seidman JG, Smith JA and Kevin S (1995). Current protocols in molecular biology. Vol 1 pp2.5.1 -2.5.17. John Wiley & Sons,Inc., USA.

Primer Designing and Validation

Santhosh J Eapen, A.Chandrasekar and O.B. Rosana

Objective

The purpose of this lab is to help you to design primers to extract Elicitin genes. We will use ClustalW to align the homologs nucleotide sequences find well-conserved regions. The next step will be to extract a conserved region from the aligned nucleotide sequences and design primers. Next step is to validate the primers using OligoCalc and FASTPCR.

Detecting Conserved regions using ClustalW

1. Access ClustalW homepage at http://www.ebi.ac.uk/Tools/clustalw2/index.html
2. Paste the sequences given below in text box and Click the Run button.
3. In the result page click start JalView Button
4. Alignment will open in an Multiple alignment editor
5. In Multiple alignment editor choose Color: percent Identity
6. Note the first conserved region.
7. Open OligoCalc at http://www.basic.northwestern.edu/biotools/oligocalc.html.
8. Enter the conserved nucleotide sequence and click Calculate button
9. Note down the primer properties, if it satisfies the GC% and Tm range.
10. Click the check self-complementarity button.
11. Check if there is any hairpin and primer dimmer formation.
12. Choose this conserved region as forward primer.
13. Choose another conserved region for reverse primer from alignment editor, such that it will have a reasonable amplicon size in between.
14. Find the reverse complement of this conserved region to get the 3' to 5' reverse primer.
15. Open molecular tool kit from http://www.vivo.colostate.edu/molkit/index.html
16. Choose Manipulate and Display Sequences. Paste the conserved region and click inverse complement button.
17. Note the reverse complement sequence and check with OligoCalc.
18. Choose this conserved region as Reverse primer.

```
>seq1

AAGTACTGTACCAGTGGTGGACCATTTCTAAATACAAATCATGGACGTGGACGCTCTCTTGTGAAGGCGT
CATTCCTCTGTTGCAGGACCAGATACGCGTCTGTCTCTCGGTCTGCCGCAGGATTCATGCCACGTGAACG
TTTTCGGTGCTTCGACAACTTTTTACAGTCGCGACGATGTCGCCGACGAAATCCAAAGAGGCAAAACCTG
TCAATGTCGTTCCAAGGCAATGCGAGTCAAGATGACATTGTTTGCTGACCCTTCCAGTCCTAGCTCCACA
ACTTCCATTGTGCAATTTGCTCTGTTATCCCCACCCACTCCTCCTCACTCCGTCCACGATGAACTTTCGT
GCTCTGTTCGCTGCCACCGTTGCTGCCCTCGTCGGCTCCACTTCCGCCACCACGTGCACCACCTCGCAGC
AGACCGTAGCGTACGTGGCGCTCGTAAGCATCCTCTCGGACACGTCGTTTAATCAGTGCTCGACGGACTC
CGGCTACTCGATGCTGACGGCCACCTCGCTGCCCACGACGGAGCAGTACAAGCTCATGTGCGCGTCGACG
GCGTGCAAGACGATGATCAACAAGATCGTGTCGCTCAACGCTCCCGACTGCGAGCTGACGGTGCCAACTA
GTGGCCTGGTACTCAACGTGTACTCGTACGCGAACGGGTTCTCGTCTACGTGTGCGTCGCTATGAGTGGA
CTTGACTCAGCGCGCTCAGCCGCTGAGTCAGCGTCCATTGGCCGTATCATCTTGTCTTTCATCGACGATT
AGGCTTTGTTCTCCGCGTCTGTTGCTATTACTGTAATAGTGTAGCAGCTGCGGTATAAAGCTATTTGAAT
AAAAATTCTTGTATGCCATACTTGTTGCTGTAGCTCGGGTGCGTTGTTTGTTCAATGCAG

>seq2

GTCGACGAAAGCCGAAGTGCGTGGCAGATCTTGCCGTTCGAATGCTACGCGCCACGGCAAAACCTACACG
GTACAACAGCTTCAAATAAACCTGCAAGCGAGCCGCCAGCCCAACTCCAGCTAGTCAAGCCTAGTTTGCC
TCCAACTGCCATTGTGCAATTTGCTCTCATCCACACCCACCCCACTTCTCCCCCACCTCATCCGAAATGA
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ACTTCCGCGCTCTGTTCGCCGCCACCGTCGCCGCCCTCGTCGGCTCCACCTCCGCCACCACGTGCACCAC
CACGCAGCAAACTGCGGCGTACGTGGCGCTCGTAAGCATCCTCTCGGACACGTCGTTCAACCAGTGCTCG
ACGGACTCTGGCTACTCAATGCTGACGGCCACCTCGTTGCCCACGACGGAGCAGTACAAGCTCATGTGCG
CGTCGACGGCGTGCAAGACGATGATCAACAAGATCGTGACGCTGAACCCGCCCGACTGCGAGTTGACGGT
GCCTACGAGCGGCCTGGTACTCAACGTGTTCACGTACGCGAACGGGTTCTCGTCTACGTGCGCGTCACTG
TAAGCGGGTTTGATCTCTGCGTCCAGAATCGAT


>seq3

ATGAACTTCCGCGCTCTGTTCGCCGCCACCGTCGCCGCCCTCGTCGGCTCCACCTCCGCCACCACGTGCA
CCACCACGCAGCAAACTGCGGCGTACATGGCGCTCGTAAGCATCCTCTCGGACACGTCGTTCAACCAGTG
CTCGACGGACTCTGGCTACTCAATGCTGACGGCCACCTCGTTGCCCACGACGGAGCAGTACAAGCTCATG
TGCGCGTCGACGGCGTGCAGGACGATGATCAACAAGATCATGACGCTGAACCCGCCCGACTGCGAGTTGA
CGGTGCCTACGGGCGGCCTGGTACTCAACGTGTTCACGTACGCGAACGGGTTCTCGTCTACGTGCGCGTC
ACTGTAA


>seq4

CGCGATCGACGGTCAACTTCAGGCTTCACCTGTGGCATGGCACCTTSGATCGAACAGTGACTGTCAGGTG
TAGACGTCAGAGAACTGAGCTGTTCGACAGCGTCTTCAGACCGCCTTTCCACTTGGTCCGAGTCAAGGTC
GCGTCGAGATCAGCCGAGCGCCCGCCACACGGGCCTACGGCTAAGGAAGGCCCTCTGCTAGGATAGCAGC
TTTTGCCAGATAGTGCGATTGCGTCTTGGGCTGTTCTGGTTCCTCTTGCTCATTCTGCAATTGGCTCCGT
CACACGCACGACATCCACTCGCCACCTCCACCCAAGATGAACTTCACCGCTCTGCTCGCTGCCGTCGCCG
CCGCCCTCGTCGGCTCCGCCAACGCCACGGCCTGCACGGCCACCCAGCAGACCGCCGCGTACAAGACGCT
CGTGAGTATCCTGTCCGAGTCGTCCTTCTCGCAGTGCTCCAAGGACTCGGGCTACTCCATGCTGACGGCC
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AGATCGTCGCGCTGAACCCTCCTGACTGCGACCTGACCGTCCCGACCAGCGGTCTCGTGCTGGACGTGTA
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GAGTTTTGAACGCGAGGCTCCCCATTGCGTACCTTCTCTCTCTGTATCGGCTAGCCATTTACTGAGGATG
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CTACGCGTTCTCTTTTGTGAGTTGGCGTGCTTGAGTGTGAGCGCACCTGCAATCACAGTTTTGACGCGGA
CCTGGCTTACCTTCGTAACAACAGAAAGCTGCCCACACTCCAGCCACGCCATCCATTTTCATTTTCGGTT
ACAGTGAATTAGTAGTTATTTGTGCTAACTACAGCCAGAGCATCAACAGGCGGCGCACTATGGGGATTCC
GGTGTTGTGCTCCTTCCATGGTTGCATCTCCGCAAGATCTTGCCGTTTCTCATTGCGTCACTTGTTGATC
CATCGAACAGCAAGAGCGCTAAGCATTTCAATGACATCTGCGTGGAATCAGCGGGGCCGTAACGATATCA
TGCCGCATCTTCGGCAAGCCGTCTAGCCATTGAGGTGCTGAACGACTGTCTTCTTGTGCTATCCGGTCTT
CAACGAGACTGCGCAACTCCAGGATCACCTGAGAGCAACAGGGAACGGACTCGCTGAGCCTGATGAAGGA
GGGGGATATGCTCGGCATGCGTCCGCTGCGACAGCTTGTCCACAGCTCCAGTCATTGCGTGCTATTGTAT
ATGACGTCTTTGTGAGCTACCGATCCGCTCACGGATACATCCTTGCCTGCGCCTACGATGCCTGCTAGAA
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CTGCAGGTGCATCACAACATAGCCGTCCATTGCCTCCGCACTCCACAATTTGAAATTTGCCTTGCCAACC
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GCTGCCACCGCCGCCGCCCTCGTCGGATCCGTCTCCGCCACGGCCTGCACTTCCACCCAGCAGACCGCCG
CCTACGTCGCGCTCGTGAGCATCCTCTCGGAGTCGTACTTCTCAACGTGCGCCAGCGACTCGGGCTACTC
CATGCTGACGGCCACGGCGCTTCCCACGACGGCTCAGTACAAGCTCATGTGCGCGTCGACCGCGTGCCAG
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AGGCACTAGCGAACGNTAGGTTGTCGCCCCCGCGCTGAAACCAGAAGAACGGGATTGTATTGTGACCAGC
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TCACTGGGGCCTCCCGAGGCAGTGCGAGAGCTGGCGACCAGGAGTGGATCTCGACCCGTTAGGCAACGGA
TCCCTTCAAGCAGCATCCATGCATCGTGGCGAGACAGCGTGGTGTGCGTGTGTGTGTGGTTATCAGGTGT
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GCCAAGCGGTTCACTGCGTGAGTCCATAGGCTACAAATGCTGCGCTGCGGCTGCGGAAGACGCGAGAAAC
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TGAAACTTTCAGGCTGAAAGACATCCTGGCGATTTCATGAGCACGAAAGATGTCTGCCGGCCGGCAAGGT
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CTACGGCGGATCCAGCCAAAATTGATCACGCTCGATCACGCTGGCAGCATGACACAGCAGCATGATGCTG
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CAAGCCACGGCAACGCCTTACCCAGAGCCATCGGCGTTAATTCACCCTCGCACCAACGCCGCCGAGTCAA
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ACGCGAACGGCTTCTCGGCCACGTGCGCGTCGCTGTAAGCAGCTTCGTTCGTGTGAGTCGCCACACTGCG
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CRCTGCTTCTCGTCCTATCCTGCCTCCGTGGTGACTGCTYCTGACACYGTGGGGCAAGAGCAGAATCGGA
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>seq5

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CGCGACGGACTCGGGCTACTCGATGCTCACGGCCACCTCGCTGCCCACGACGGCGCAGTACAAGCTCATG
TGCGCGTCGACGGCGTGCAACACCATGATCACCAAGATCGTGTCGCTGAACCCCACCCGACTGCGAGCTGA
CGGTGCCGACCAGCGCCCTGGTGCTCAACGTGTACTCGTACGCGACGCACGGCTTCTCGACCACGTGCGCGTC
GCTGTAAGCGACTTGGTTCGTGGCAATCCCCACGTTCAGCGCATCCACCCCCAAGACGTGGACTCGTCTT
CAGATCAAGTACGCATCTTGAGCATTGTTTCAGCATTTATAAGGCTTTTGAATGAAATAAGTTTTTGTTT
CCGAAAAAAAAAAAAAAA

>seq6

ATGAACTTCCGCGCTCTGTTCGCCGCCACCGTCGCCGCGCTCGTCGGCTCCACCTCGGCCACCACGTGCA
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CGCGACGGACTCGGGCTACTCGATGCTCACGGCCACGTCGCTGCCCACGACGGCGCAGTACAAGCTCATG
TGCGCGTCGACGGCGTGCAACACCATGATCACCAAGATCGTGTCGCTGAACCCGCCCGACTGCGAGCTGA
CGGTGCCGACCAGCGGCCTGGTGCTCAACGTGTACTCGTACGCGCACGGCTTCTCGACCACGTGCGCGTC
GCTGTAAGCAGCTTGGTTCGTGGCAATCCCCCATCCACCCTCAAGACGCAGACTCACCTCTTATGCCGAC
TTGTCTTCAGATCAAGTACGCAGATTGAGCAGGTAGTGTTGGCGTCGGGCGTGCATTTAACTGTTGTTTG
AACATTTATAAGGCTTTTGAATGAGCAAGTTTTTGTTTCCGAAAAAAAAAAAAAAA

>seq7

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CGACCACCCAGCAGACCGCCGCGTACGTCGCCCTGGTGAGCATCCTCTCGGACTCGTCGTTCAACCAGTG

```
CGCCACGGACTCTGGCTACTCGATGCTGACGGCCACGGCGCTGCCCACGACGGCCCAGTACAAGCTCATG
TGCGCGTCGACGGCGTGCAACACGATGATCACCAAGATCGTGTCGCTGAACCCCCCTGACTGCGAGCTGA
CGGTGCCCACAAGCGGTCTGGTGCTCAACGTTTACTCGTACGCGAACGGTTTCTCGTCTACGTGCGCGTC
ACTGTAA
```

Exercise II

Identify primers for the following gene sequence using Primer3 and Primer3 plus and check the properties using OligoCalc.

1. Access Primer3 at http://frodo.wi.mit.edu/primer3/
2. Paste the sequence below
3. Set the product size range between 1500-1700.
4. Click pick primer button.
5. Check for primer properties using OligoCalc.

```
GACAAGAACCTGGAGATCAAGGTGACCCCGGACAAGGCCAACGGCACGCTGACCATCCAGGACTCTGGTA
TTGGTATGACGAAGGCTGACCTGATTAACAACCTGGGTACGATCGCCAAGTCGGGCACCAAGGCCTTCAT
GGAGGCTCTGGCTGCTGGCGCCGACATCAGTATGATTGGTCAGTTCGGTGTGGGTTTCTACTCGGCCTAC
CTGGTCGCTGACAAGGTCGTGGTGCACTCGAAGCACAACGATGACGAGCAGTACGTGTGGGAGTCTGCTG
CCGGTGGCTCGTTCACTGTCACGCCTGACACGTCGGAGCCCATCCAGCGCGGTACGCGCATTGTGCTGAA
GCTCAAGGAGGACATGCTCGAGTACCTTGAGGAGCGCAAGCTTAAGGACCTGGTGAAGAAGCACTCGGAG
TTCATTGGCTTCCCCATYAAGCTGTACGTCGAGAAGACGGAGGAGAAGGAAGTGACGGACGACGAGGAAG
AGGAGGACGAGAAGGAGGGCGAGGACGACAAGCCCAAGGTCGAGGAGGTCGAAGAGGAGGAAGGCGAGAA
GAAGAAGAAGACTAAGAAGATTAAGGAAGTGACCCACGAGTGGGACCACCTCAACAGCCAGAAGCCCATC
TGGATGCGCAAGCCTGAAGACGTGACCCACGAGGAGTACGCGTCGTTCTACAAGTCGCTGACGAACGACT
GGGAGGAGCACGCCGGCGTCAAGCACTTTTCGGTGGAGGGCCAGCTGGAGTTCAAGGCYTGTCTGTTCAC
CCCCAAGCGCGCGCCGTTCGACATGTTYGAGGGCGGCGCCAAGAAGAAAGTGAACAACATCAAGCTGTAC
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TCGTCGACTCGGAGGACTTGCCGCTTAACATCTCGCGTGAGACGCTGCAGCAGAACAAGATCCTGCGTGT
GATCAAGAAGAACCTGGTCAAGAAGTGCCTCGAGATGTTCGCCGAACTGGCTGAGGAYAACGAGAAGTAC
AACAAGTTCTACGAGTCGTTCAGCAAGAACCTCAAGCTGGGCATCCACGAGGACTCGACCAACCGCACCA
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TCGCCATTCATTGAGAAGCTCAAGAAGAAGGGCTACGAGGTGCTGTTCATGGTGGAGGCCATTGACGAGT
ACGCTGTGCAGCAGCTCAAGGAGTACGAGGGCAAGAAGCTCATCTGCGCCACCAAGGAGGGCCTCAAGAT
GGAGGAGACGGAGGACGAGAAGAAGTCGTTCGAGGAGCGCAAGGCTGCCACTGAAGGACTGTGCAAGCTC
ATGAAGGAGGTGCTGGACGACAAGGTGGAGAAGGTGGAGATCTCGAACCGTATTGTGGAGTCGCCCTGTG
TGCTTGTTACCGGCGAGTACGGCTGGTCGGCCAACATGGAGCGCATYATGAAGGCGCAGGCTCTGCGTGA
CAGCAGCACGTCGGCCTACATGTCGTCCAAGAAGACGATGGAGATCAACCCGCTGCACCCTATTATCAAG
TCGCTGCGCGAGAAGGCGGAGGCTGACAAGAGCGACAAGACGGTCAAGGACCTCATCTGGCTGCTG
```

Exercise III

Identify SSR for the sequence below using MISA and make out the flanking primers using Primer3

1. MISA - MIcroSAtellite identification tool. This tool allows the identification and localization of perfect microsatellite.
2. Copy the sequence below and save in .fasta format in MISA folder.
3. In terminal window change the directory to MISA by typing the following command:
   cd MISA
4. In MISA folder open misa.ini and set the parameters as below and save the file

definition(unit_size,min_repeats): 1-10 2-10 3-6 4-6 5-6 6-6
interruptions(max_difference_between_2_SSRs): 100

5. Run the program by typing the following command:

./misa.pl <filename.fasta>

Output:

Results of the microsatellite search are stored in two files:

6. In "<FASTfile>.misa" the localization and type of identified microsatellite(s) are stored in a tablewise manner.
7. The file "<FASTfile>.statistics" summarizes different statistics as the frequency of a specific microsatellite type according to the unit size or individual motifs.

To design flanking Primers

8. Access Primer3 at http://frodo.wi.mit.edu/primer3/
9. Paste the sequence below
10. Set the product size range between 250-300
11. Set the target position (E.g. 704, 24 requires primers to surround the 24 bases at positions 704)
12. Click pick primer button.
13. Check for primer properties using OligoCalc.

```
ACCACCTACTACTCAAAGCAAGCAACGAACTCAAGGCTACTTCAGCCCACCCAAACCAAA
GACTCTCACCAAGACTCTCTACCGACCATGTTCAGCTCGTGGGATCTCGTCCGACCGCGC
TTCTGGTACCCCATGTCGTCACTGGAACAGTCCATGATGGAGCTCGAGCACATGAGCGAC
CTCATGAGGCGCTCGCGCTTCCCGTTCGACACGAGCAACGAGATGTTGGCGGCACCCAGC
AGCCACGACGACGACGACTTCTTCCGGGACCTCCCAGTGCTGGCGCGCGAGCAACAACCA
GCGGCACAGCAACGCGAGAAGGGAGCACAGCCATCGCCAGTGGACAAGCAGACGGTCGCA
GCAACCAATACCACCCGGGACAATGACCCGAATAAACAGACCAACGAGAACGATCATGTC
CACCGTCGCGCCTTCTCGTCCTACTCCTTCAGCAACTCCTCCGTCGTGGACGACAAAGGC
CGACGTGTGACGAGCACGCGCCGTCGCTACGAAGACTCGACCGGTCGCCTTAAAGCCGTG
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GATAAGGGCCAGCACGAGTCCGTGTGTTCGAGCGGCTCGCCCGACGAGTTCGAGGCGCTG
TGGCAGCAGACGCCCTTCGGTGCAGCACAGAAGAAGACCATCAAGCAGCAGCAGCAGCAG
CAGCAGCAGTTGGAGCAAGGACAGCAGAGTCAACAGGAGAAGTCCCCAACCAGCTCGAAA
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GACGGCAGTAACGCAAGCAGCCAACGCGAAGCCTTGTCGCCCATTTGCTATCGACTGCGG
GGTGAAACTTCCCAGTACCGCCAACTGCGTGAAAATGTGCAAACACGACGAAGGAGCCGA
TACGAGGGTGAAGCTCAGCAACGTACACAAGTCGTTCGTCGTTCTACATACATGAGTAAA
GAGTAATAAACAACTATTTAGTTTCCAAACCAC
```

Exercise IV

1. Do *in silico* **PCR** in **FASTPCR** for identifying the PCR product size and binding sites.

2. Paste the target sequence and paste the primers click the run button find the PCR product size.

Primers:
```
>
ACGGAGCCTCCTTTTTCTTC

>
TGTCCTCATGGGAGCACATA
```

Seq:

>Phytophthora

```
CCTTGGCCTCTGCCTAATCACACAGATTCTAACAGGATTATTTCTCGCAATACACTACACAGCTGACA
TCTCAACAGCCTTCTCCTCCGTCGCCCACATCTGTCGAGATGTTAACTACGGATGACTAATTCGAAAC
ATTCATGCAAACGGAGCCTCCTTTTTCTTCATCTGCCTCTACCTTCACGTAGCCCGAGGCATATACTA
TGGCTCATACCTCTACAAAGAAACCTGAAACATCGGAGTAGTTCTCCTACTCCTAACTATAATAACCG
CCTTCGTAGGATATGTGCTCCCATGAGGACAGATATCCTTCTGAGGAGCCACCGTAATTACCAACCTT
CTTTCCGCCTTCCCCTACATCGGGGACACCCTAGTACAATGAATCTGAGGTGGTTTCTCAGTAGACAA
CGCCACCCTAACC
```

>Ralstonia

```
CCTAGGCCTCTGCCTTATTACACAAATCTTAACAGGACTATTTCTTGCAATACACTACACAGCTGACA
TTTCAACAGCCTTCTCCTCCGTCGCCCACATCTGCCGAGACGTAAACTACGGGTGACTAATCCGAAAC
GTCCACGCAAATGGCGCCTCCTTCTTCTTTATCTGCTTGTACCTTCACGTCGCACGAGGTATATACTA
CGGCTCCTACCTCCAAAAAGAAACCTGAAACATCGGAGTAGTCCTCTTACTCCTCACCATAATAACCG
CCTTCGTAGGCTATGTACTGCCCTGAGGACAAATATCATTTTGAGGGGCAACCGTAATCACTAACCTC
CTTTCCGCCTTCCCGTACATCGGCGACACATTAGTGCAATGAATCTGAGGCGGCTTTTCAGTC
```

Molecular approaches for studying the genetic diversity of fungal pathogens

M Anandaraj

Genetic diversity – a molecular approach

Genetic diversity of microorganisms particularly of beneficial microbes is important for selecting the most suitable one for effective biocontrol of plant diseases. Molecular markers are useful tools for developing detailed linkage maps of species or even at lower taxonomic levels among isolates of a single species from different populations (which are likely to be similar and to have overlapping traits) suitable distinguishing feature which may be difficult or impossible to find out using conventional methods. Molecular markers, however, can be applied at these levels with great reliability and they allow simultaneous measurement of variability at multiple loci of each individual tested.

Genetic Markers: Isozymes

Isozymes are alternative enzyme forms encoded by different alleles at the same locus, which can be used as informative genetic markers. Multiple polymorphic loci can be surveyed and typically 2 or 3 are detected at each locus. Randomly sampled Isozymes loci are generally accepted to be of independent genetic origin. However, Isozyme variations in many pathogenic fungi are low or Non-existent.

DNA based markers:

RFLP: These are the first DNA based markers developed. They may be the result of length mutation and /or point mutation at a restriction digests of genomic DNA, cDNA or mitochondrial DNA fragments from specific DNA segments amplified using PCR. Thus, depending on the probe used, RFLPs can be used to analyze mtDNA variation, ribosomal (r)DNA region variation, repetitive and single-copy sequence variations. RFLPs are co-dominant markers. This makes them suitable for population genetic studies as well as for linkage map construction. By employing probes that detect multiple loci and dispersed repetitive sequences, the sensitivity of the RFLP can be enhanced to fingerprinting resolution.

AFLP

RFLPs can be converted to AFLPs by ligating adaptors for PCR amplification. The method offers the potential to detect large numbers of amplification products. Although this method does not target specific areas of the genome for marker identification, the large number of loci that can be analyzed in a single experiment greatly improves the chance of identifying markers linked to the chosen locus

PCR with Arbitrary primers

Direct Amplification Fingerprinting (DAF) (Caetano- Anolles *et al,* 1991) involves the use of short primers, five to eight nucleotides long, with low or high stringency annealing steps and a two-temperature instead of standard three temperature cycling program. Resulting fragments are separated on polyacrylamide gels and visualized by silver staining. AP-PCR (Welsh and McClelland, 1990) is performed with oligonucleotides with 20 or more nucleotides, were used as primers. Two cycles with low stringency (allowing for mismatches) were followed by 3- to 40 cycles with high stringency. This method is employed to assay variation "within species" (Williams *et al.*, 1990), in which arbitrary short oligonucleotide primers, target unknown sequences in the genome, are used to generate amplification products that often show size polymorphism within species.RAPD analysis offers the possibility of creating polymorphisms without any prior knowledge of the DNA sequences of

the organism investigated. The patterns produced are highly polymorphic, allowing discrimination between isolates of a species if sufficient numbers of primers are screened. RAPDs data can contain artifacts are not fully reproducible. However, RAPDs have been used to generate large numbers of genetic markers useful for linkage mapping quickly and cheaply.

Uses:
RFLP analysis is a laborious and time-consuming procedure that requires previous information of the genome. All these limitations can be overcome by RAPD analysis.

RAPD is useful in generating large numbers of DNA markers for species whose genomes are relatively unknown.Specific primer designing: RAPD fragments can be used to clone and sequence data, to design PCR primers specific for particular organism which then can function as sequence tagged sites (STSs). This method eliminates the problems associated with reproducibility associated with RAPD analysis.

Primer Screening
Primer screening is performed to ascertain the suitability of primers and to select primers for the RAPD analysis. For screening primers PCR reactions are set up using DNA from model organism as template. Label 0.2ml PCR tubes.
1. Prepare reaction stock mixture. (Eg. For 10 reactions prepare stock for 11 Reactions) Add all the components except primers.
2. Dispense 24μl reaction mixture in each PCR tube.
3. Add 1μl of selected primers to each different tube.
4. Keep the tubes in the thermal cycler and run the machine using desired program.

PCR with template DNA as variable
For the RAPD analysis, the primers giving positive amplification during screening are selected. These selected primers are used to amplify DNA from all the 22 isolates. In this reaction, the template DNA is a variable while all other reaction constituents are kept constant.
Procedure:
1. Label twenty-two 0.2-ml PCR tubes.
2. Prepare reaction stock mixture according to table 1. Add each component in the given order except the template DNA.
3. Dispense 24μl of stock in each PCR tube.
4. Add 1μl of respective template to each tube.
5. Place the tubes into the thermal cycler and amplify DNA using the required conditions.

Post PCR analysis of data
Resolve the PCR products in 1.4% agarose gel using protocols mentioned earlier and record the results for analysis using suitable programme for data interpretation

Molecular approaches for studying the genetic diversity of *P. capsici* black pepper

Culturing of *P. capsici*: *Phytophthora capsici*, the foot rot pathogen of black pepper is used for DNA isolation. To ensure the genetic purity the single zoospore cultures are plated on the *Phytophthora* specific medium (PVPH incorporated Carrot Agar) and after 48 hours the mycelial disc is transferred into the carrot agar medium and after the period of incubation of 24-48 hrs the mycelial disc is aseptically transferred into the liquid medium (GYP).

DNA isolation from *Phytophthora*

1. 0.1gm of four days old mycelium + 750μl odf SDS extraction buffer + 50mg of polyvinyl polypyrolidone + 10mg of glass powder
2. Grind well with minigrinder
3. Centrifuge at 13,000 rpm for 5 minutes
4. Add mixture of phenol:chloroform:isoamyl alcohol (25:24:Supernatant added with the 1) to supernatant and mix well
5. Centrifuge at 13,000 rpm for 5 minutes
6. Add isopropanol (2/3 vol)
7. Centrifuge at 13,000 rpm for 10 minutes.
8. Look for DNA pellet and wash the pellet in 70% ethanol by centrifuging at 13,000 rpm for 2 minutes.
9. Dry the pellet and add 50μl of sterile distilled water with Rnase. (20ug/ml)

RFLP PCR

ITS6 and ITS4 were used to identify different species of *Phytophthora*. The primer sequence for ITS 6 is 5'GAAGGTGAAGTCGTAACAAGG 3' and ITS4 is 5' TCCTCCGCTTATTGATATGC 3'.

The PCR mixture contained

DNA	1.0μl (50ng)
Taq pol (1U)	0.5μl
Reaction buffer (1X)	2.5μl
$MgCl_2$ (2mM)	3.0μl
dNTP (100μM)	2.0 μl
Primes ITS4 and ITS6	1.25μl +1.25μl (each primer)
Water to makeup	25μl

The PCR machine is programmed as follows.

1. Initial denaturation at 94°C for 3 minutes, 40 cycles of denaturation at 94°C for 1 minute, followed by annealing at 55°C for 1 minute and polymerization at 72°C for 2 minutes and final extention at 72° C for 10 minutes
2. Restriction digestion of the amplified product
3. Once the reaction is over, out of 25μl, 5 μl is used to check out the presence of the amplified product of 862bp by agarose gel electrophoresis of 1.4%. Once the presence of the product is confirmed, out of the remaining 20μl was separated into 10 μl each, and the first 10μl is subjected to MSP restriction digestion by adding
4. 0.5μl of restriction enzyme (Msp I or Taq I)
5. 0.2μl of restriction enhancer (BSA),
6. 2μl of 10X restriction buffer and
7. 7.3μl of sterile distilled water, making up the total volume of 20μl.
8. This was subjected to incubation at 37°C for 2 hrs.
9. The same is done with the left out 10μl of the PCR product but with the change in the restriction enzyme, i.e instead of Msp I, Taq I (restriction enzyme) is used and incubated at 65°C for 2 hrs.
10. And the restricted product of Msp I and Taq I are electrophoresed in 2.4% agarose gel, and is documented.

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ITS-rDNA ANALYSIS OF PHYTOPHTHORA

Advancements in molecular methods have permitted a more rational study of phylogenetic relationships within various organisms. Among the assortment of genetic markers that have been used for oomycete phylogeny, the rapidly evolving, noncoding internal transcribed spacer (ITS) regions of the ribosomal DNA seem to be one of the most popular choice primarily because of their relatively high sequence variability. These lie between two coding regions, the 18S and the 28S genes. Another coding region, the 5.8S gene, is found between the ITS1 and ITS2.

Materials required
Genomic DNA
2.5mM dNTPs mix
Primer (ITS-6 & ITS-4)
Taq DNA polymerase
PCR reaction buffer
$MgCl_2$ (25mM)
Deionized water
PCR tubes and tips and
Materials required for agarose gel electrophoresis

Procedure
Set up PCR reaction on ice as follows: Supposing that we have 10 samples, prepare a PCR master mix on ice as follows:

10X PCR buffer	25μl
$MgCl_2$ (25mM)	15μl
dNTPs mix (2.5mM)	5μl
ITS-6 (10pmole)	12.5μl
ITS-4 (10pmole)	12.5μl
Taq polymerase (5U/μl)	2.5μl
Deionized water	167.5μl
Total reaction	240μl

Take 10 PCR tubes and aliquot 24μl of this master mix to each tube. Now add 1μl template DNA to each tube so that the total reaction volume becomes 25μl.

Perform PCR using the following cycling conditions:

Initial denaturation	94°C for 3 minutes	
Denaturation	94°C for 30 seconds	
Annealing	55°C for 30 seconds	34 cycles
Extension	72°C for 1 minute	
Final extension	72°C for 10 minutes	
Hold	4°C for 5 minutes	
End		

Analysis of the PCR products using agarose gel electrophoresis

1. Prepare a 1.0% agarose gel.
2. Add 5μl of 6X loading dye to 25μl of PCR amplified product and mix well.
3. Load each sample in a well.
4. Load 3μl of 500bp ladder molecular weight marker to estimate the size of the amplified product. The average size of the product is around 900bp.
5. Run the gel at 80V for 90 minutes.
6. Document the image in Gel documentation system.

Molecular characterization of bacteria

A. Kumar

The identification and classification of symbiotic and pathogenic plant-associated bacteria are important both in terms of their agricultural applications, as well as for basic studies on plant-microbe interactions. A number of different phenotypic and genotypic methods are presently being employed for microbial identification and classification. Each of these methods permits a certain level of phylogenetic classification, from the genus, species, subspecies, biovar to the strain specific level. Moreover, each method has its advantages and disadvantages, with regard to ease of application, reproducibility, requirement for equipment and level of resolution (Akkermans *et al.* 1995).

A variety of phenotypic methods has been traditionally used to type bacteria, including serotyping, phage typing, microscopic identification, substrate utilization screening (e.g. BIOLOG), multilocus enzyme electrophoresis (MLEE), fatty acid methyl ester analysis (FAME), 2-D PAGE of total proteins, and intrinsic antibiotic resistance profiling. Majority of these techniques requires purification and cultivation of the bacteria or can be quite laborious and time consuming (Hook *et al.* 1991). The resulting taxonomy, however, does not necessarily reflect phylogeny, relationships by evolutionary descent. Most microbiologists would prefer to have taxonomic schemes based on phylogeny since the grouped bacteria should share close genetic backgrounds and thus common phenotypes. Phylogenetic analysis can be based on the amino acid sequence of proteins and on the presence of similar metabolic pathways (two early methods), but the most accurate method of determining phylogenetic relationships is the comparison of DNA composition and sequence.

Restriction Fragment-Length Polymorphism (RFLP): With the discovery of restriction endonucleases, rapid methods were devised to identify and cluster bacteria by what has been called Restriction Endonuclease Analysis (REA) and Restriction Fragment-Length Polymorphism (RFLP) analysis. Genomic DNA from most bacteria when digested with "6-basepair cutters" like *Eco*RI, produces about 1000 DNA fragments of varying sizes that can be analyzed (separated) by agarose gel electrophoresis. The collection of restriction endonuclease-generated DNA fragments from a given species or strain of bacteria gives a distinctive pattern when analyzed by gel electrophoresis, and this pattern can be used to differentiate bacteria or strains of bacteria. This whole-genomic-pattern method is but one example of REA. RFLP analysis is a technique used mainly to detect genetic variation in a single gene. A polymorphism (difference in fragment size) of a specific restriction endonuclease fragment has to be "linked" to a specific genetic allele. The fragment size is monitored by gel electrophoresis, and the specific fragment is usually identified by Southern blotting and probing the DNA pattern with a labeled DNA fragment that hybridizes only to the one, specific fragment. The shortcomings of both REA and RFLP analysis is that they again are slow labor intensive and usually require a large amount of DNA.

Random Amplification of Polymorphic DNA (RAPD): RAPD analysis is a PCR based molecular marker technique. Here single short oligonucleotide primers are arbitrarily selected to amplify a set of DNA segments distributed randomly throughout the genome. RAPD amplification is performed in condition resembling those of polymerase chain reaction using genomic DNA from the species of interest and a single short oligonucleotide (primer). The DNA amplification product is generated from a region which is flanked by a part of 10 bp priming sites in the appropriate orientation. Genomic DNA from two different individuals often produces different amplification patterns. A particular fragment generated for one

individual but not for other represents DNA polymorphism and can be used as a genetic marker. RAPD reactions are PCR reactions, but they amplify segments of DNA, which are essentially unknown to the scientist (random). Often, PCR is used to amplify a known sequence of DNA. Thus, the scientists chooses the sequence he or she wants to amplify, then designs and makes primers which will anneal to sequences flanking the sequence of interest. Thus, PCR leads to the amplification of a particular segment of DNA However, in RAPD analysis, the target sequence(s) (to be amplified) is unknown. The scientist will design a primer with an arbitrary sequence. In other words, the scientist simply makes up a 10 base pair sequence (or may have a computer randomly generate a 10 bp sequence), and then synthesizes the primer. The scientist then carries out a PCR reaction and runs an agarose gel to see if any DNA segments were amplified in the presence of the arbitrary primer. The primers must anneal in a particular orientation (such that they point towards each other). The primers must anneal within a reasonable distance of one another

Interpretations

- ✓ RAPDs exhibit polymorphism and thus can be used as genetic markers.
- ✓ RAPDs are dominant in the sense that the presence of a RAPD band does not allow distinction between hetero- and homozygous states.
- ✓ RAPD markers linked to genes of interest can serve as starting points for chromosome walk to isolate those genes.
- ✓ RAPD protocols are relatively simple.
- ✓ In some cases, RAPD bands of differing lengths can be assigned to the same locus. In that case, these RAPD bands are codominant

Rep-PCR: Versalovic *et al*. (1994) described a method for fingerprinting bacterial genomes by examining strain-specific patterns obtained from PCR amplification of repetitive DNA elements present within bacterial genomes. Two main sets of repetitive elements are used for typing purposes. The repetitive extragenic palindromic (REP) elements are 38-bp sequences consisting of six degenerate positions and a 5-bp variable loop between each side of a conserved palindromic stem (Stern *et al.* 1984.) REP sequences have been described for numerous enteric bacteria (Gilson *et al.* 1984; Hulton *et al.* 1991). The palindromic nature of the REP elements and their ability to form stem-loop structures have led to multiple proposed functions for these highly conserved dispersed elements (Gilson *et al.* 1990; Yang and Ames 1988).

Rep-PCR can be performed with DNA extracted from bacterial colonies or by a modified method using unprocessed whole cells (Woods 1993). REP or ERIC amplification can be performed with a single primer, a single set of primers, or multiple sets of primers. ERIC patterns are generally less complex than REP patterns, but both give good discrimination at the strain level. Application of both REP and ERIC PCR to samples to be typed increases the discriminatory power over that of either technique used alone. Rep-PCR is fast becoming the most widely used method of DNA typing. The technique is easy to perform and can be applied to large or small numbers of isolates. Rep-PCR shows broader species applicability and better discriminatory power than either plasmid profiling or genomic fingerprinting (Georghiou 1995.). Rep-PCR has considerably better discriminatory power than restriction analysis of the 16S rRNA gene or the 16S-23S-spacer region (Appuhamy 1997). Furthermore,

studies which have compared Rep-PCR to other typing methods such as multilocus enzyme electrophoresis, biochemical characterizations, or ribotyping (Vila 1996). A comparative study of different PCR-based DNA fingerprinting techniques for typing has shown Rep-PCR to be superior to these methods.

ERIC PCR: The enterobacterial repetitive intergenic consensus (ERIC) sequences are a second set of DNA sequences which have been successfully used for DNA typing. ERIC sequences are 126-bp elements that contain a highly conserved central inverted repeat and are located in extragenic regions of the bacterial genome (Sharples and Lloyd. 1990). They have been defined primarily based on sequence data obtained from *E. coli* and *Salmonella typhimurium.*

BOX PCR: While the REP and ERIC sequences are the most commonly used targets for DNA typing, another repetitive element, the BOX sequence, has been used to differentiate bacteria (Koeuth *et al.* 1995). BOX elements are located within intergenic regions and can also form stem-loop structures due to their dyad symmetry. They are mosaic repetitive elements composed of various combinations of three subunit sequences referred to as boxA, boxB, and boxC (Martin *et al*. 1992) The three-subunit sequences have molecular lengths of 59, 45, and 50 nucleotides, respectively. The BOX elements have no sequence relationship to either REP or ERIC sequences (Martin *et al.* 1992). While initially thought to be unique to *S. pneumonia*, BOX elements have now been found in a number of bacterial species

Amplified Ribosomal DNA Restriction Analysis (ARDRA): The conserved nature of the rRNA sequences has also led to the development of generic DNA probes for bacteria. The amplified ribosomal gene (rDNA) is subjected to restriction endonuclease digestion this has been termed ARDRA (Amplified Ribosomal DNA Restriction Analysis). The resulting restriction fragment pattern is then used as a fingerprint for the identification of bacterial genomes. This method is based on the principle that the restriction sites on the RNA Operon are conserved according to phylogenetic pattern (Woese 1987; Stackebrandt and Goebel 1994). The 16S rRNAs are valuable phylogenetic marker molecules for microorganisms because they are universally distributed and constant in function and because different positions of their sequences change at very different rates. This makes the 16S rRNA gene suitable for many analytical methods, like sequence analysis, ribotyping, restriction fragment length polymorphism (RFLP) analysis, and hybridization with oligonucleotide probes, all useful for the identification and typing of microorganisms. Of all these methods, RFLP analysis of PCR generated rDNA fragments named ARDRA (amplified rDNA restriction analysis) is used very common.

Conclusion

Clearly, the utility of DNA-based approaches has been enhanced tremendously by the application of PCR. A most useful, application of PCR to bacterial identification and classification has been in the area of genomic fingerprinting. Different PCR-based DNA fingerprinting techniques were used for the identification of bacteria. The PCR-based DNA fingerprinting techniques were: (i) repetitive extragenic palindromic (REP) PCR; (ii) enterobacterial repetitive intergenic consensus (ERIC) PCR; (iii) BOX PCR; (iv) randomly amplified polymorphic DNA (RAPD); (v) restriction analysis of the amplified 16S rRNA gene (ARDRA-16S); and (vi) restriction analysis of an amplified region containing the 16S-23S rRNA spacer region and part of the 23S rRNA gene (ARDRA 23S + spacer).

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Cloning and identification of recombinants

A.I. Bhat, A. Siljo and E.J. Suraby and I.P. Vijeshkumar

The term clone refers to a large number of cells/molecules identical with a single ancestral cells or molecule. Cloning is a process where a foreign DNA is inserted into a carrier DNA molecule (called as vector) using the recombinant DNA technology and such recombinant DNAis then transferred into a suitable host system where it can replicate vigorously and thus generates the copy number of the target DNA molecule. Recombinant DNA technology involves removing, modifying or adding stretch of DNA to another DNA. It is possible to cut DNA strands into segments using restriction enzymes. The fragments so generated can be joined with similarly cut DNA molecule. Thus recombinant DNA molecule is a chimeric DNA molecule where different DNA molecule from different sources is joined together. Basic steps of DNA cloning involve (i) isolation of target DNA (ii) selection of suitable vector where the target DNA will be inserted (iii) joining the target DNA with the vector (ligation) (iv) transfer of the recombinant DNA into a suitable host (transformation) (v) selection of recombinant cones (screening).

Different types of vectors such as plasmids, bacteriophage, cosmid, phagemid, Fosmid, bacterial artificial chromosome (BAC) and year artificial chromosome (YAC) are available for cloning. Of all, plasmid vectors are highly suitable for cloning PCR products. Plasmid are extrachromosomal, covalently closed, circular double stranded DNA molecules which can autonomously replicate within a bacterial cell. Plasmids usually contain one or two antibiotic resistance genes which helps for selection of recombinant plasmids. Ligation of a DNA with another DNA is facilitated by the enzyme called DNA ligase. DNA ligase joins 5´ phosphate (P) group to 3´hydroxyl (OH) group of DNA through the formation of covalent phosphodiester bands. The catalytic activity of the enzyme requires the presence of ATP and Mg++.

Transformation involves the process of introducing the ligated recombinant plasmid into bacteria. Transformation has been described in many species of bacteria that have the ability to take up DNA naturally. Only a small percentage of a bacterial population has this ability and these few cells are said to be competent. In *E. coli* competent cells do not occur naturally. Hence plasmid DNA do not enter *E. coli* cells under their own power, but require assistance traversing the outer and inner cell membranes and in reaching an intracellular site where they can be expressed and replicated. This can be achieved by using competent bacterial cells for transformation. Bacterial cells can be made competent (to take up foreign DNA) either by chemical or physical methods. Most of the chemical methods are based on the observations of Mendel and Higa (1970) who showed that bacteria treated with ice cold solutions of $CaCl_2$ and then briefly heated to 37^oC or 42^oC could be transfected with bactriophage lamda DNA. The same method was later used to transform bacteria with plasmid DNA. The physical method known as elctrophoration involve exposure of bacterial cells to electrical charge that destabilizes their membranes and thus induces the formation of transient membrane pores through which DNA molecules can pass. Although electrophoration is easy fast and efficient, it is expensive requiring costly electrical equipment and specially designed cuvettes.

There are three basic methods used for cloning a PCR product. The include: (i) TA cloning: Since the PCR product generated by *Taq* polymerase is appended with a single extraneous dA at 3' ends, the easiest way of cloning is by using plasmid tailed with dT. (ii) Blunt end cloning: The blunt ended PCR product generated by *Pwo* or *Pfu* polymerase can be cloned into a plasmid restricted with blunt end generating enzymes. (iii) Directional cohesive end cloning: In this case PCR product is first restricted with appropriate restriction enzymes

followed by ligating them onto plasmid linearized by same restriction enzymes. In all the above methods PCR products are first purified to remove enzymes, unused primers, dNTPs etc. For this the PCR product is first run on low melting agarose gel, the band of interest is excised and purified. Alternatively PCR product can also be purified using commercial kits. The purified PCR product is then ligated with linearized dT or blunt end or cohesive end vector using *E.coli* T$_4$ DNA ligase. If directional cloning is planned, restriction of PCR product is carried out with appropriate enzymes before ligation with vector. The ligated vector is then used to transform competent *E.coli* cells and transformants are selected using appropriate markers such as antibiotic or blue / white colony morphology.

The following protocol describes cloning using TA vector.

i) Purification of PCR product (will be done using commercial kit, Procedure may vary depending on the kit used)

ii) Ligation of the purified PCR product into vector

Requirements

Materials
Eppendorf tubes
Ice flakes
Microcentrifuge
Micropipette
Microtips
Refrigerator

Reagents
10x T4 DNA ligase buffer
T4 DNA ligase
Linearized TA vector (Commercially available)
Purified PCR product
Sterile water

Procedure

1. Set up ligation reaction with purified PCR product in a TA plasmid vector.
    10x T4 DNA ligase buffer   1.0 µl
    Linearized TA vector (20 ng/µl)   1.0 µl
    Purified PCR product (10 ng/µl)   7.0 µl
    T4 DNA ligase (3 Weiss units/µl)   1.0 µl
    Mix the reactants by pipetting and incubate at 4°C for 24 h.

2. Ligated plasmid is now ready for transformation.

iii) Preparation of competent *E.coli* cells

Requirements

Materials
Autoclave
Balance

Centrifuge tubes
Conical flask
Eppendorf tubes
Glasswares
High speed centrifuge
Ice bucket
Ice flakes
Incubator
Inoculation needle
Laminar flow
Marker pen
Micropipette
Microtips
Refrigerator
Shaker incubator
Sterile petriplats

Reagents
E.coli bacterial strain (DH 5 ☐)
Luria broth (for preparation see appendix)
Luria agar (for preparation see appendix)
0.1 M $MgCl_2$ (for preparation see appendix)
0.1 M $CaCl_2$ (for preparation see appendix)
Sterile water

Procedure
1. Pick a single bacterial colony from a freshly prepared plate and transfer into 100 ml broth. Incubate the culture with vigourous shaking (200 rpm) at 37^oC till the O.D reaches 0.4 at 600 nm.
2. When the required O.D is reached, transfer the bacterial cells to sterile disposable, ice cold 50 ml polypropylene tubes. Store on ice for 10 min.
3. Centrifuge the cells at 2700 g for 10 min at 4^oC.
4. Decant off the supernatant and resuspend the pellet by swirling in 30 ml of ice cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$, 20 mM $CaCl_2$).
5. Recover the cells by centrifugation as above.
6. Decant the medium from pellets and resuspend the pellet in 2 ml of ice cold 0.1M $CaCl_2$ for each 50 ml of original culture.
7. At this point cells achieve competence. The cells can be directly used for transformation or can be dispersed into aliquots and frozen at -70^oC for later use.

 (iv) Transformation of *E.coli*

Requirements

Materials
Eppendorf tubes
Ice flakes
Incubator cum shaker
Laminar flow
Microcentrifuge
Micropipette
Microtips
Spreader

Water bath

Reagents
Appropriate antibiotics solution
Competent cells
IPTG (Isopropyl β-D-1-thiogalctopyranoside) (0.1 M) 24 mg/ml in water, filter sterilized and stored at -20 ^{o}C
Luria agar: Luria broth + agar (15 g/l)
Luria broth
    Bacto-tryptone 10g
    yeast extract 5g
    NaCl: 10g
    Dissolve and make up the volume to 1000 ml
Recombinant ligated plasmid
X-gal (2%): Dissolve 20 mg in 1 ml of dimethyl formamide (DMF). Store at -20 ^{o}C, protected from light
(For reagent preparation, see appendix)

Procedure
1. Take 200 µl of competent cells in a sterile chilled tube. Add about 50 ng recombinant plasmid (ligation mix) in a volume of 10 µl or less. Also, keep a control for transformation, by incubating 2 ng of plasmid with 200 µl of competent cells. Have one extra tube of cells as untransformed control to check for contamination.
2. Mix gently and incubate on ice for 30- 45 min.
3. Transfer the tubes to 42°C in a water bath for 90-120 sec (to give heat shock to cells).
4. Quickly transfer tubes to an ice bath and chill for 1-2 min.
5. Add 1 ml of LB medium and incubate at 37°C for 1 h with shaking (at 200 rpm).
6.   Plate the cells on appropriate selective medium (Luria agar containing x-gal, IPTG and appropriate antibiotics) using different volumes (200 µl, 400 µl, 500 µl). Spread uniformly with the help of spreader.
7. Incubate the plate inverted overnight at 37°C and check for the transformants next morning.

Screening of recombinant plasmids based insertional inactivation of marker gene
The inactivation of a gene occurs by inserting a fragment of DNA into the middle of the coding sequence. Any future products from the inactivated gene will not work because of the extra codes added to it. The *lacZ* gene has been used in prokaryotic cloning vectors as a marker for insertion of the gene of insert. Typically, when transformations are plated on medium containing the substrate X-gal, colonies which contain vector with an insert appear white while those that contain vector without an insert are blue. The hydrolysis of x-gal by β-galactosidase causes the characteristic blue colour in the colonies. It shows that colonies contain self ligted vector. White colonies indicate insertion of foreign DNA and loss of the cells ability to hydrolyse the marker (x-gal). In some cases, however, light blue colonies or white colonies with blue centres will appear. These clonies often contain vector with inserts

Screening and identification of positive clones

From the mater plate, positive recombinant clones can be identified by any one or combination of methods given below:
          (i)   Colony PCR using insert or combination of vector and insert specific primers
          (ii)   Isolation of recombinant plasmid and release of insert by restriction digestion

    (iii)   Isolation of recombinant plasmid and PCR using vector and insert specific primers

    (iv)   Sequencing of insert

v) Isolation of recombinant plasmid DNA

Many methods are available to purify plasmids from bacteria. These methods involve: growing of bacterial culture, harvesting and lysis of bacteria and purification of plasmid DNA. Bacteria are harvested from late logarithmic phase grown cultures by centrifugation. The supernatant is removed from cell pellets. Many methods such as nonionic detergents, organic solvents, alkali and heat are available to lyse the bacterial cells. The choice depends on the size, strain of *E. coli* and technique used subsequently to purify DNA. Among all alkali lysis method is largely used.

Alkaline lysis method (Brinboin and Doly, 1974)

Requirements

Materials
Balance
Beakers
Horizontal gel apparatus and power pack
Ice flakes
Magnetic stirrer
Measuring cylinders
Microcentrifuge
Microcentrifuge tubes
Micropipette
Microtips
Microwave oven
pH meter
Shaking waterbath
Transilluminator

Reagents
Master plate containing putative transformants
Agarose
Ethidium bromide
Solution I:
20% Glucose ------2.25 ml
0.5M EDTA, pH 8.0---------1.00 ml
Sterile distilled water ------ 45.50 ml

Solution II
10N NaOH   0.4 ml
10% SDS   2.0 ml
Sterile distilled water   18.6 ml
(not be autoclaved)

Solution III
3M sodium acetate pH4.8. Dissolve 40.81 g of sodium acetate.$3H_2O$ in 60 ml water. Adjust pH to 4.8.

Solution IV

1M Tris pH 8.0   2.50 ml
3M NaoAC pH 4.8   1.65 ml
Sterile water   45.85ml

RNase A: Dissolve pancreatic RNase A at a concentration of 10 mg/ml in 10 mM Tris-HCl pH 7.9 and 15mM NaCl. Heat to 100°C for 15 min and allow to cool slowly to room temperature. Dispense into aliquots and store at -20°C.

TE, pH 8.0: Tris base 0.1211 g; EDTA 0.0372 g. Adjust ph to 8.0 with HCl. Adjust volume to 100 ml.

Phenol: chloroform: isoamylalcohol (25:24:1)
Ethanol
TAE buffer

Procedure

1. Select colonies which are positive in Rapid and colony PCR and inoculate them individually in 5 ml-broth with appropriate antibiotic at 37°C for 14 h with shaking (200rpm).
2. Collect the growth in a 1.5 ml eppendorf tube and spin for 30 sec in microcentrifuge, pour off supernatant
3. Suspend pellet thoroughly in 100 µl of solution I by vortexing immeadiately. Keep on ice for 5 min.
4. Add 200 µl freshly prepared solution II at room temperature. Mix gently by inverting tubes (do not vortex) and keep on ice for 5 min or until SDS precipitates. Samples should be viscous and stringly when cap opened at completion of this step.
5. Add 150 µl solution III at room temperature and mix gently by inverting tubes several times. Keep on ice for 5 min.
6. Spin 3 min in centrifuge in cold room for 5 min at 14,000 rpm at 4°C. Without disturbing the pellet, carefully remove the supernatant and put into a fresh eppendorf tube. Discard the pellet.
7. Add 0.9 to 1.0 ml cold ethanol. Mix gently and freeze at -70°C for 15 min.
8. Spin in centrifuge for 2-3 min. Remove and discard supernatant.
9. Add 100 µl solution IV to the pellet and gently mix till pellet is dissolved.
10. Add 200 µl cold ethanol and freeze at -70°C for 15 min again.
11. Spin in microfuge for 2-3 min and remove the supernatant. Wash pellet with chilled 70% ethanol to remove excess salts. Dry final pellet for 5 min under vacuum.
12. Suspend pellet in 20 µl of TE and store at -20°C until needed.

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Sequence Alignment

Santhosh J Eapen, O.B. Rosana and A. Chandrasekar

Multiple sequence alignment (MSA)

Introduction
A multiple sequence alignment (MSA) is a sequence alignment of three or more biological sequences, generally protein, DNA, or RNA. In many cases, the input set of query sequences are assumed to have an evolutionary relationship by which they share a lineage and are descended from a common ancestor. From the resulting MSA, sequence homology can be inferred and phylogenetic analysis can be conducted to assess the sequences' shared evolutionary origins. Visual depictions of the alignment as in the image at right illustrate mutation events such as point mutations (single amino acid or nucleotide changes) that appear as differing characters in a single alignment column, and insertion or deletion mutations (indels or gaps) that appear as hyphens in one or more of the sequences in the alignment. Multiple sequence alignment is often used to assess sequence conservation of protein domains, tertiary and secondary structures, and even individual amino acids or nucleotides.

Multiple sequence alignment also refers to the process of aligning such a sequence set. Because three or more sequences of biologically relevant length can be difficult and are almost always time-consuming to align by hand, computational algorithms are used to produce and analyze the alignments. MSAs require more sophisticated methodologies than pairwise alignment because they are more computationally complex. Most multiple sequence alignment programs use heuristic methods rather than global optimization because identifying the optimal alignment between more than a few sequences of moderate length is prohibitively computationally expensive

Tool used
ClustalW2 is a general purpose multiple sequence alignment program for DNA or proteins.

Objectives
- To find out the homologous sequence and conserved region in multiple sequences.

1. **Find out the homologous sequences in *Oryza sativa, Triticum aestivum, Hordeum vulgare, Secale cereal & Zea mays* by performing BLASTp for the sequence given below and execute their MSA using clustalW and note down the conserved regions.**
  >Seq

MGFAPMLSVAVLLGTLAAFPAAVHSIGVCYGVVANNLPGPSEVVQLYRSKGIDSMRI
YFADAAALNALSGSNIGLIMDVGNGNLSSLASSPSAAAGWVRDNIQAYPGVSFRYIA
VGNEVQGSDTANILPAMRNVNSALVAAGLGNIKVSTSVKFDRFADTFPPSNGRFRDE
YMTPIAKFLATTGAPLLANVYPTLPTKRNRKAGRKTSCFNYATFQPGRTVVDNGNRL
TYTCFCDAMVDSIYAALEKAGTPSVSVVVSESGWPSAGGKVGASVNNAQTYNQGLI
NHVRGGTPKKRRALETNIFAMFDENGKPGDEIEKHFGLFNPNKSPSYSISF

2. **Download the sequences with the following accession numbers AF030167, AK248899, AK331482, BLYGLU2X and AB244642 from NCBI, perform MSA and view their score table and study the percentage similarity of each pair of sequences.**
(i) Which sequences are most closely related? Support your answer with specific data.
(ii) What is the score between BLYGLU2X and AB244642

(iii) Open the alignment in Jalview- Java multiple alignment editor and color by percentage identity.

Phylogenetic Analysis:

A phylogenetic tree or evolutionary tree is a branching diagram or "tree" showing the inferred evolutionary relationships among various biological species or other entities based upon similarities and differences in their physical and/or genetic characteristics. The taxa joined together in the tree are implied to have descended from a common ancestor. In a **rooted** phylogenetic tree, each node with descendants represents the inferred most recent common ancestor of the descendants, and the edge lengths in some trees may be interpreted as time estimates. Each node is called a taxonomic unit. Internal nodes are generally called hypothetical taxonomic units (HTUs) as they cannot be directly observed. Trees are useful in fields of biology such as bioinformatics,systematics and comparative phylogenetics.

1. Draw a phylogenetic tree for the following sequences using clustal W program.
    a) Download the fasta file for following accession No from NCBI – HC730879, NM_001178103 , HM173354, NM_200174, GU992202. (Arabidopsis, Cattle, Ecoli, zebra fish, Human)
    b) Paste the sequences in multiple seq alignment program clustalW and run the analysis.
    c) Find the phylogenetic tree at the bottom of the results.
    d) Which two sequences appear to be most closely related by viewing the unrooted tree?
    e) Click show as phylogram button and show distance button to find the distance for Human.
2. Construct a phylogenetic tree for the following sequences using MEGA5 program.

Example D-loop hypervariable regions from five primate species.

> Homo sapiens neanderthalensis
GTTCTTTCATGGGGGAGCAGATTTGGGTACCACCCAAGTATTGACTCACCCATCA
GCAACCGCTATGTATCTCGTACATTACTGTTAGTTACCATGAATATTGTACAGTAC
CATAATTACTTGACTACCTGCAGTACATAAAAACCTAATCCACATCAAACCCCCC
CCCCCATGCTTACAAGCAAGCACAGCAATCAACCTTCAACTGTCATACATCAACT
ACAACTCCAAAGACGCCCTTACACCCACTAGGATATCAACAAACCTACCCACCCT
TGACAGTACATAGCACATAAAGTCATTTACCGTACATAGCACATTACAGTCAAAT
CCCTTCTCGCCCCCATGGATGACCCCCCTCAGATAGGGGTCCCTTGAT
>neanderthal
CCAAGTATTGACTCACCCATCAACAACCGCCATGTATTTCGTACATTACTGCCAG
CCACCATGAATATTGTACAGTACCATAATTACTTGACTACCTGTAATACATAAAA
ACCTAATCCACATCAACCCCCCCCCCCCATGCTTACAAGCAAGCACAGCAATCAA
CCTTCAACTGTCATACATCAACTACAACTCCAAAGACACCCTTACACCCACTAGG
ATATCAACAAACCTACCCACCCTTGACAGTACATAGCACATAAAGTCATTTACCG
TACATAGCACATTATAGTCAAATCCCTTCTCGCCCCCATGGATGACCCCCCTCAG
ATAGGGGTCCCTTGA
>Human
TTCTTTCATGGGGAAGCAGATTTGGGTACCACCCAAGTATTGACTTACCCATCAA
CAACCGCTATGTATTTCGTACATTACTGCCAGCCACCATGAATATTGCACGGTAC
CATAAATACTTGACCACCTGTAGTACATAAAAACCCAATCCACATCAAAACCCCC
TCCCCATGCTTACAAGCAAGTACAGCAATCAACCCTCAACTATCACACATCAACT
GCAACTCCAAAGCCACCCCTCACCCACTAGGATACCAACAAACCTACCCACCCTT
AACAGTACATAGTACATAAAGCCATTTACCGTACATAGCACATTACAGTCAAATC
CCTTCTCGTCCCCATGGATGACCCCCCTCA

> Gorilla

TTCTTTCATGGGGAGACGAATTTGGGTGCCACCCAAGTATTAGTTAACCCACCAA
TAATTGTCATGTATGTCGTGCATTACTGCCAGCCACCATGAATAATGTACAGTAC
CACAAACACTCCCCCACCTATAATACATTACCCCCCCTCACCCCCCATTCCCTGCT
CACCCCAACGGCATACCAACCAACCTATCCCCTCACAAAAGTACATAATACATAA
AATCATTTACCGTCCATAGTACATTCCAGTTAAACCATCCTCGCCCCCACGGATG
CCCCCCTTCAGATAGGGATCCCTTAAACACCATCCTCCGTGAAATCAATATCCCG
CACAAGAGTGCTACTCTCCTCGCTCCGGGCCCATAACACCTGG

>chimpanzees

GTACCACCTAAGTATTGGCCTATTCATTACAACCGCTATGTATTTCGTACATTACT
GCCAGCCACCATGAATATTGTACAGTACTATAACCACTCAACTACCTATAATACA
TTAAGCCCACCCCCACATTACAACCTCCACCCTATGCTTACAAGCACGCACAACA
ATCAACCCCCAACTGTCACACATAAAATGCAACTCCAAAGACACCCCTCTCCCAC
CCCGATACCAACAAACCTATGCCCTTTTAACAGTACATAGTACATACAGCCGTAC
ATCGCACATAGCACATTACAGTCAAATCCATCCTTGCCCCCACGGATGCCCCCCC
TCAGA TAGG

>orangutans

TTCTTTCATGGGGGACCAGATTTGGGTGCCACCCCAGTACTGACCCATTTCTAAC
GGCCTATGTATTTCGTACATTCCTGCTAGCCAACATGAATATCACCCAACACAAC
AATCGCTTAACCAACTATAATGCATACAAAACTCCAACCACACTCGACCTCCACA
CCCCGCTTACAAGCAAGTACCCCCCCATGCCCCCCCACCCAAACACATACACCGA
TCTCTCCACATAACCCCTCAACCCCCAGCATATCAACAGACCAAACAAACCTTAA
AGTACATAGCACATACTATCCTAACCGCACATAGCACATCCCGTTAAAACCCTGC
TCATCCCCACGGATGCCCCCCCTCAGTTAGTAATCCCTTACTCACCATCCTCCG

    a) Open MEGA5 program click Align and choose Edit/Build Alignment, check the create new alignment give OK. It will ask DNA or Protein give DNA, Alignment explorer will open paste the sequences.

    b) Go to Alignment tab choose align by clustalW give OK. Now sequences are aligned.

    c) Then click the Data tab choose phylogenetic analysis it will ask protein coding give yes and proceed.

    d) Go to main program click phylogeny choose construct/test Maximum Likelihood tree. Give compute to construct tree.

    e) Same way do for Neighbor joining and UPGMA also.

    f) Find out which is the most related to HUMAN ?

    g) Find out the branch length (distance) for all ?

3. Analyze DNA, protein seq and construct tree using MEGA5 program.

Expressed Sequence Tag Analysis

Santhosh J Eapen, A. Chandrasekar and O.B. Rosana

EST clustering and assembly

Currently the majority of the coding portion is in the form of expressed sequence tags (ESTs), and the need to discover the full length cDNAs of each human gene is frustrated by the partial nature of this data delivery. There is significant value in attempting to consolidate gene sequences as they are produced, in lieu of a yet-to-be-completed reference sequence. ESTs offer a rapid and inexpensive route to gene discovery, reveal expression and regulation data, highlight gene sequence diversity and splicing, and may identify more than half genes of organisms. Unfortunately, most EST data remains unprocessed, and thus does not provide the important high value sequence consensus information that it contains. The low quality sequence data provided can be much improved on, and in order to achieve quality information, pre-processing, clustering and post-processing of the results is required. The steps for EST processing are given below.

Exercise 1. Collection of ESTs.

NCBI dbEST is a division of **GenBank** that contains sequence data and other information on "single-pass" cDNA sequences, or "Expressed Sequence Tags", from a number of organisms. For downloading the complete EST sequences of organisms of your interest type the scientific name of organism in the text box, available EST sequences of organism of your interest can be obtained

1. Go to NCBI site http://www.ncbi.nlm.nih.gov/ select db EST and type *Phytophthora capsici* in text box.
2. Observe the results and download the fasta format file for analysis.
   (**FASTA format** is a text-based format for representing either nucleotide sequences or peptide sequences, in which base pairs or amino acids are represented using single-letter codes. The format also allows for sequence names and comments to precede the sequences. Which begins with '>', and then give a name and/or a unique identifier for the sequence)
3. By selecting the display format as fasta will provide the fasta file.
4. By selecting file, whole ESTs can be downloaded in a single file.

Exercise 2: Vector Screening

Downloaded EST's may contain vector and poly A tail contaminations, these vector sequences and poly A tail sequences must be removed to avoid errors during annotation. The vector screening step will show you whether your EST sequences contain Vector contamination.

1. Go to http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html for removing vector contamination.
2. Copy and paste your fasta file in to the text box.
3. Click Run VecScreen button.
4. Find the similarities using Vector Blast. If similarities found delete the similar sequence from the fasta file.
5. Use the preprocessed file for further analysis.

Exercise 3: TrimEST

If the vector sequence is detected during vector screening, use TrimEST tool to trim out the vector sequences present in the EST.

6. Access http://inn.weizmann.ac.il/cgi-bin/EMBOSS/emboss.pl?_action=input&_app=trimest for removing PolyA tail.
7. Browse and choose your fasta file or paste your EST sequence.
8. Observe the options field manipulate it and click run trimEST. Immediately output file will open.
9. For larger size sequences submit mail ID so that you will get a mail your job is over or not.

Exercise 4: TrimSeq – Trim ambiguous bits off the ends sequences. Specifically, it removes all gap characters from the ends, removes X's and N's (in nucleic sequences) from the ends, optionally removes *'s from the ends, optionally removes IUPAC ambiguity codes from the ends (B and Z in proteins, M, R, W, S, Y, K, V, H, D and B in nucleic sequences). It then optionally trims off poor quality regions from the end, using a threshold percentage of unwanted characters in a window which is moved along the sequence from the ends. The unwanted characters which are used are X's and N's (in nucleic sequences), optionally *'s, and optionally IUPAC ambiguity codes.

1. Go to http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=trimseq.
2. Check file option and browse and choose your file click the RUN button to trim the sequence.
3. Save the TrimSeq output file for further analysis.

Exercise 5. Repeat masking

Repeat masking is not a necessary step; this tool is used to mask the repeated regions of EST, which may create problems for clustering algorithm for EST assembly.

1. RepeatMasker - screens DNA sequences in FASTA format against a library of repetitive elements and returns a masked query sequence ready for database searches.
2. Go to the website http://repeatmasker.org in services click repeatmasking
3. Browse and choose your fasta file or paste your EST sequence.
4. Check search engine as wublast and choose the DNA source.
5. Result output will be in HTML format.
6. This repeat masked file is used for clustering.

Exercise 6. Clustering and Assembling the ESTs

A cluster is fragmented, EST data (DNA or protein) and (if known) gene sequence data, consolidated, placed in correct context and indexed by gene such that all expressed data concerning a single gene is in a single index class, and each index class contains the information for only one gene. Clustering refers to assembling sequences in specific order as such; they were placed in the genome of organism.

1. Go to CAP3 website http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=cap3 and type your Email ID.
2. Paste the EST sequences in the text box or upload the file by selecting file option.
3. Click the run button.
4. CAP3 will make the EST sequence in to contigs and singletons.
5. Once analyzed, save the contigs file and singleton file. These contigs and singleton were used to further analysis.

EST Annotation

Genome or EST annotation is the process of attaching biological information to sequences. It consists of two main steps:

1. Identifying elements on the genome, a process called gene prediction, and
2. Attaching biological information to these elements.

The basic level of annotation is using BLAST for finding similarities, and then annotating genomes based on that. However, nowadays more and more additional information is added to the annotation platform. The additional information allows manual annotators to deconvolute discrepancies between genes that are given the same annotation. Some databases use genome context information, similarity scores, experimental data, and integrations of other resources to provide genome annotations through their Subsystems approach. Other databases (e.g Ensembl) rely on both curated data sources as well as a range of different software tools in their automated genome annotation pipeline.

Structural annotation consists of the identification of genomic elements.

- ORFs and their localization
- gene structure
- coding regions
- location of regulatory motifs

Functional annotation consists of attaching biological information to genomic elements.

- biochemical function
- biological function
- involved regulation and interactions
- expression

These steps may involve both biological experiments and *in silico* analysis. A variety of software tools have been developed to permit scientists to view and share genome annotations.

Steps

Exercise 1. The clustered EST sequence obtained from EST clustering and assembly step is used for annotation of EST

Exercise 2. Annotation of ESTs using blast.

1. Go to BLASTX site (Search **protein** database using a **translated nucleotide** query) http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Translations&PROGRAM=blastx&BLAST_PROGRAMS=blastx&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on and paste your contigs sequence for blast search.
2. Type the organism as *Phytophthora* with default parameters.
3. Observe the search results.
4. In Blast results click the gene ontology GO and observe the function of the gene.
5. Prepare the table for the functional annotation.
6. Interpret your results.

Exercise 3. Annotation of ESTs using ESTEXPLORER

1. Go to ESTEXPLORER (Web server) site and observe the interface for EST analysis (http://estexplorer.els.mq.edu.au/estexplorer/main_page.php).
2. Select organism as *Phytophthora*
3. Check EST sequences and upload your data.
4. Tick PHASE I , PHASE II, PHASE III
5. Provide your name and mail ID
6. Click Process data button.
7. It will provide the request ID to see the results via status of the work.

RNA Isolation (Plants)

Jhonson K George and I.P. Vijeshkumar

Introduction

Obtaining high quality, intact RNA is the first and often the most critical step in performing many fundamental molecular biology experiments, including Northern analysis, nuclease protection assays, RT-PCR, RNA mapping, in vitro translation and cDNA library construction. To be successful, however, the RNA isolation procedure should include some important steps both before and after the actual RNA purification.

Research into optimizing RNA analysis has identified two points in the RNA isolation process that can be improved; treatment and handling of tissue or cells prior to RNA isolation and storage of the isolated RNA. Since most of the actual RNA isolation procedure takes place in a strong denaturant (e.g. GITC, LiCl, SDS, phenol) that renders RNases inactive, it is typically prior to, and after the isolation, when RNA integrity is at risk.

The protocol outlined here is a modification of the RNA isolation protocol of Chomczynski and Sacchi (1987)

Reagents required
  - Liquid nitrogen
  - Denaturing buffer (4M Guanidine thiocyanate, 25mM Sodium citrate, 1% sarcosyl)
  - Acid phenol
  - Chloroform: isoamyl alcohol (24:1)
  - Plant RNA isolation aid (ambion)
  - β-mercapto ethanol
  - RNA Later
  - Cold isopropanol
  - Cold absolute alcohol
  - 75% ethanol
  - RNA Storage solution
  - 0.1% DEPEC treated water

Equipments
  - Pre cooled Mortar and pestle
  - Refrigerated centrifuge
  - Oak ridge tubes
  - Gel electrophoresis unit
  - Power pac
  - Vortex mixer
  - Deep freezer
  - Ice flaker
  - Eppendorf tubes
  - Phase lock gel
  - Spectrophotometer

Protocol

GTC-PHENOL METHOD FOR RNA ISOLATION:
  1. Grind two grams of leaves in liquid nitrogen

2. Add ground leaf material to fresh phase lock gel containing 5 ml denaturing buffer and 100 μl of β- mercapto ethanol .

3. Add 0.5 ml 2M sodium acetate (pH 4) and mix by inverting the tube.

4. Add 5 ml acid phenol and mixed well by inverting.

5. To the above mixture add one ml of chloroform:isoamyl alcohol (24:1) and one ml of plant RNA isolation aid (Ambion) mixed well and the tubes incubated on ice for 30 minutes.

6. Centrifuge at 10000 rpm for 20 minutes at 4°C.

7. Carefully transfer the supernatant to a fresh phase lock gel.

8. To the supernatant add equal volume of acid phenol:chloroform:isoamylalcohol (1:1)

9. Centrifuge at 10000rpm for 10 minutes

10. Transfer the supernatant to a fresh oak ridge tube.

11. Add an equal volume of cold isopropanol , mixed and incubated at-20 °C for one hour.

12. Centrifuge at 10000 rpm for 20 min at 4 °C.

13. Dissolve the pellet in 1.5 ml denaturing buffer and distribute 0.5 ml in three microfuge tubes.

14. Add equal volume of cold 100% ethanol or isopropanol to each tube and incubate at-20 °C for 30 minutes.

15. Centrifuge at 10000 rpm for 15 minutes at 4 °C and discard the supernatant

16. Resuspend RNA pellet in 75% ethanol and incubate at room temperature for 15 minutes and centrifuge for 10 minutes at 10000 rpm Cat and aspirate the supernatant.

17. Air dry the pellet for 15 minutes and dissolve in nuclease free water or RNA storage solution.

Preparation of denaturing buffer:

Guanidine thiocyanate (4M)	47.26 g (MW 118.2)
Sodium citrate (pH 7.0) 25mM	0.735g (MW 294.1)
Sarcosyl 1%	1.0 g

Mix and dissolve the above reagents in 100 ml of DEPC treated water and use after autoclaving.

2 M Sodium acetate (pH 4) MW 82.03:

Dissolve 164.06 g of sodium acetate in 800 ml of DEPC treated water. Adjust the pH to 4.0 with glacial acetic acid. Adjust the volume to 1 litre with DEPC water and use after autoclaving.

Chloroform: isoamyl alcohol: (24:1)
Mix 96 ml of chloroform with 4 ml of isoamyl alcohol

Procedure for DEPC treatment of water
0.1% DEPC treated water is prepared by dissolving 1 ml DEPC in 1000ml of distilled water and stirring continuously for 30 minutes. This is then autoclaved and used for preparing reagents.

DEPC Treatment of equipments glasswares , plastic wares etc
All glasswares , plastic wares, pestle and mortar immersed in 0.1% DEPC treated water, kept overnight and then autoclaved.

Gel preparation for RNA
1.2 g of agarose is boiled in 72 ml DEPC treated water and 10 ml of 10XMOPS and 18 ml of formaldehyde added and the gel is cast.

RNA sample treatment and electrophoresis
 RNA -4-8 µL
 10X MOPS-2.0 µL
 Formaldehyde-4 µL
 Formamide-10 µL
The samples heated at 60 degrees for 5 minutes cooled on ice for 5 minutes and 4 µL of 5x loading dye is added and the samples run at 80V for one hour
Analysis of RNA quality with a spectrophotometer
 The total RNA quantified with a spectrophotometer at 230, 260, 280 nm.

Protocol for RT-PCR

FOR 1ST STRAND SYNTHESIS

RNA	8µL (200-250 ng)
Oligo dT	1µl (0.5µg/µl)
Water	3.75 µl
Total	12.75 µl

Mix and centrifuge the above contents and incubate at 65 degrees in water bath for five minutes and then chill for five minutes, and add

5X cDNA synthesis buffer	4 µl
RNase inhibitor	0.25 µl (2500 units)
dNTP	2.0 µl (10mM)
M-MULV Reverse Transcriptase-	1.0 µl (200 units)
Total	7.25 µl
Total reaction volume	20 µl

Incubate the tubes at 42 degrees for 60 minutes and 70 degrees for 10 minutes to inactivate the reverse transcriptase.

Second strand synthesis

cDNA	1 µl (from the first strand product)
10X Taq Buffer	2 µl
dNTP	1 µl (10mM)
Primer Forward	1 µl (10pM)
Primer Reverse	1 µl (10pM)
TAQ polymerase	0.3 µl (5Units/ µl)
Sterile water	13.7 µl
Total	20.0 µl

PCR conditions:
94**°**C- 2 minutes
94**°**C- 30 seconds
Annealing temperature according to Tm of the primer
72**°**C- 1 minute
72**°**C- 10 minutes
4**°**C- hold

Gel electrophoresis of the PCR products
The second strand products run on 1.2% agarose gel in 1X TBE Buffer. Document the gel using Kodak gel logic system.

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Real-Time PCR

D. Prasath and T.P. Prameela

The real-time polymerase chain reaction uses fluorescent reporter dyes to combine DNA amplification and detection steps in a single tube format. The increase in fluorescent signal recorded during the assay is proportional to the amount of DNA synthesised during each amplification cycle. Individual reactions are characterised by the cycle fraction at which fluorescence first rises above a defined background fluorescence, a parameter known as the threshold cycle (Ct) or crossing point (Cp). Consequently, the lower the Ct, the more abundant the initial target. This correlation permits accurate quantitation of target molecules over a wide dynamic range, while retaining the sensitivity and specificity of conventional end-point PCR assays. The homogeneous format eliminates the need for post-amplification manipulation and significantly reduces hands-on time and the risk of contamination. Real-time PCR is often abbreviated to qPCR, although that abbreviation is not universally accepted.

Real-Time PCR Applications

Real-Time PCR can be applied to traditional PCR applications as well as new applications that would have been less effective with traditional PCR. With the ability to collect data in the exponential growth phase, the power of PCR has been expanded into applications such as:

- Viral Quantitation
- Quantitation of Gene Expression
- Array Verification
- Drug Therapy Efficacy
- DNA Damage measurement
- Quality Control and Assay Validation
- Pathogen detection
- Genotyping

Advantages of using Real-Time PCR:

- Traditional PCR is measured at End-Point (plateau), while Real- Time PCR collects data in the exponential growth phase
- An increase in Reporter fluorescent signal is directly proportional to the number of amplicons generated
- The cleaved probe provides a permanent record amplification of an Amplicon
- Increase dynamic range of detection
- No-post PCR processing
- Detection is capable down to a 2-fold change

Real-Time chemistries allow for the detection of PCR amplification during the early phases of the reaction. Measuring the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection. Traditional methods use Agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction.

Real-Time PCR Evolution
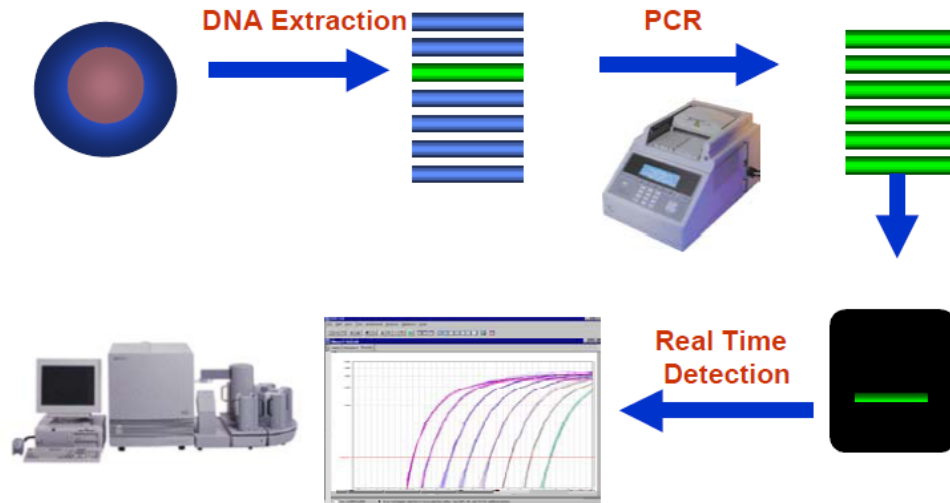
Real-Time chemistries allow for the detection of PCR amplification during the early phases of the reaction. Measuring the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection. Traditional methods use Agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction.

Limitations of End-Point PCR
Agarose gel results are obtained from the end point of the reaction. Endpoint detection is very time consuming. Results may not be obtained for days. Results are based on size discrimination, which may not be very precise. As seen later in the section, the end point is variable from sample to sample. While gels may not be able to resolve these variabilities in yield, real-time PCR is sensitive enough to detect these changes. Agarose Gel resolution is very poor, about 10 fold. Real-Time PCR can detect as little as a two-fold change.

Some of the problems with End-Point Detection
- Poor Precision
- Low sensitivity
- Short dynamic range < 2 logs
- Low resolution
- Non - Automated
- Size-based discrimination only
- Results are not expressed as numbers
- Ethidium bromide for staining is not very quantitative
- Post PCR processing

As you can see from the figure, the samples in the gel contain 10 copies and 50 copies, respectively. It is hard to differentiate between the 5-fold change on the Agarose gel. Real-Time PCR is able detect a two-fold change (i.e. 10 vs. 20 copies).
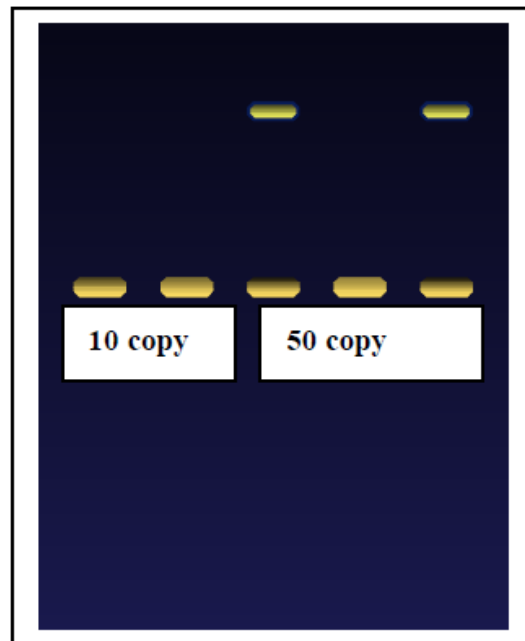
PCR Phases:

To understand why end-point PCR is limiting, it is important to understand what happens during a PCR reaction. A basic PCR run can be broken up into three phases:

Exponential: Exact doubling of product is accumulating at every cycle (assuming 100% reaction efficiency). The reaction is very specific and precise.

Linear (High Variability): The reaction components are being consumed, the reaction is slowing, and products are starting to degrade.

Plateau (End-Point: Gel detection for traditional methods): The reaction has stopped, no more products are being made and if left long enough, the PCR products will begin to degrade.
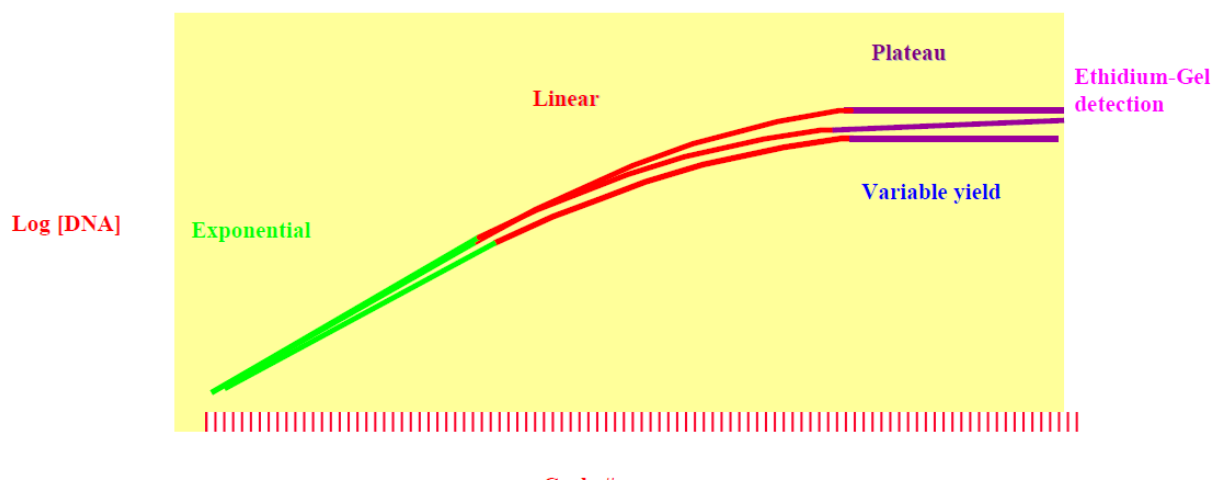
The above figure shows three replicates of a sample. The replicates have the same starting quantity. As the PCR reaction progresses, the samples begin to amplify in a very precise manner. Amplification occurs exponentially, that is a doubling of product (amplicon) occurs every cycle. This type of amplification occurs in the exponential phase. Exponential

amplification occurs because all of the reagents are fresh and available, the kinetics of the reaction push the reaction to favor doubling of amplicon.

However, as the reaction progresses, some of the reagents are being consumed as a result of amplification. This depletion will occur at different rates for each replicate. The reactions start to slow down and the PCR product is no longer being doubled at each cycle. This linear amplification can be seen in the linear phase of the reaction. The three samples begin to diverge in their quantities during the linear phase.

Eventually the reactions begin to slow down and stop all together or plateau. Each tube or reaction will plateau at a different point, due to the different reaction kinetics for each sample. These differences can be seen in the plateau phase. The plateau phase is where traditional PCR takes its measurement, also known as end-point detection.

Figure also shows that the three replicate samples, which started out at the same quantity in the beginning of the reaction; reflect different quantities at the plateau phase. Since the samples are replicates they should have identical quantities. Therefore, it will be more precise to take measurements during the exponential phase, where the replicate samples are amplifying exponentially. The amplification phases can be viewed differently to assess the PCR phases. The figures that follow show the phases of PCR in a Logarithmic scale view and a Linear scale view.

Problems with detection in the Plateau phase of PCR
The following three figures show the plateau affect on 96 replicates and a fivefold dilution series. As stated earlier, the plateau region is the end-point of the reaction and is representative of the amount of product that you would see on Agarose Gels. The 96 replicates in the exponential phase are very tight in both the linear and logarithmic views. In the logarithmic view, the plateau for each reaction seems to occur in the same place, but this is solely due to the log scaling of the plot. Figure 6 shows the same 96 replicates in linear view. The reactions show a clear separation in the plateau phase; therefore, if the measurements were taken in the plateau phase, quantitation would be affected.

Real-Time chemistry provides fast, precise and accurate results. Real-Time PCR is designed to collect data as the reaction is proceeding, which is more accurate for DNA and RNA quantitation and does not require laborious post PCR methods.

Quantitation
Theoretically, there is a quantitative relationship between amount of starting target sample and amount of PCR product at any given cycle number. Real-Time PCR detects the accumulation of amplicon during the reaction. The data is then measured at the exponential phase of the PCR reaction. Traditional PCR methods use Agarose gels or other post PCR detection methods, which are not as precise. As mentioned earlier, the exponential phase is the optimal point for analyzing data. Real-Time PCR makes quantitation of DNA and RNA easier and more precise than past methods.

Quantification strategies
Absolute quantification
Standard curve with known (mRNA / DNA). 100 % efficient assays may be highly sensitive

Relative quantification
Compare amount using a reference – normalization. If using the 2–ΔΔCT (Livak) method or the ΔCT method using a reference gene, it's only possible to compare assays with similaror very high PCR efficiency. If PCR efficiency is taken into account (Pfaffl method), more variation in PCR efficiency is tolerated

There are three main chemistries in general use:

Intercalating dyes, such as SYBR-Green, which fluoresce upon light excitation when bound to double stranded DNA. These are cheap, easily added to legacy assays and amplification products can be verified by the use of melt curves. They can lack specificity and fluorescence varies with amplicon length. In general, they are one Ct or so more sensitive than probe-based assays.

Fluorophores attached to primers, e.g. Invitrogen's Lux or Promega's Plexor primers. These are relatively inexpensive and amplification products can be verified by melt curves. Specificity depends on the primers and specific, usually company-specific design software needs to be used for optimal performance. This is not necessarily a bad thing (indeed the Plexor software is very useful), but it is not always possible to change primer design parameters.

Hybridisation-probe based methods, e.g. hydrolysis (TaqMan) or Molecular Beacons. These are the most specific, as products are only detected if the probes hybridise to the appropriate amplification products. There are many variations on this theme, with melt curve analysis possible for some chemistries. Their main disadvantages are cost, complexity and occasional fragility of probe synthesis. There are potential problems associated with the fact that probe-based assays do not report primer dimers that can interfere with the efficiency of the amplification reaction.

The 5' Nuclease Assay
In the 5' nuclease assay, an oligonucleotide called a TaqMan® Probe is added to the PCR reagent master mix. The probe is designed to anneal to a specific sequence of template between the forward and reverse primers. The probe sits in the path of the enzyme as it starts to copy DNA or cDNA. When the enzyme reaches the annealed probe the 5' exonuclease activity of the enzyme cleaves the probe.

SYBR Green Dye
SYBR Green chemistry is an alternate method used to perform real-time PCR analysis. SYBR Green is a dye that binds the Minor Groove of double stranded DNA. When SYBR Green dye binds to double stranded DNA, the intensity of the fluorescent emissions increases. As more double stranded amplicons are produced, SYBR Green dye signal will increase. SYBR Green dye will bind to any double stranded DNA molecule, while the 5' Nuclease assay is specific to a pre-determined target.

The increase in reporter signal is captured by the Sequence Detection instrument and displayed by the software. The Figure below shows an increase in the reporter signal over time. The amount of reporter signal increase is proportional to the amount of product being produced for a given sample. When the fluorescent signal Reporter increases to a detectable level it can be captured and displayed as an Amplification Plot, The Amplification Plot contains valuable information for the quantitative measurement of DNA or RNA. The Threshold line is the level of detection or the point at which a reaction reaches a fluorescent intensity above background. The threshold line is set in the exponential phase of the amplification for the most accurate reading. The cycle at which the sample reaches this level is called the Cycle Threshold, Ct. These two values are very important for data analysis using the 5' nuclease assay.

Rn: measure of reporter signal

Threshold is the point of detection.

Cycle-Threshold (Ct) cycle at which sample crosses threshold

Cycle #
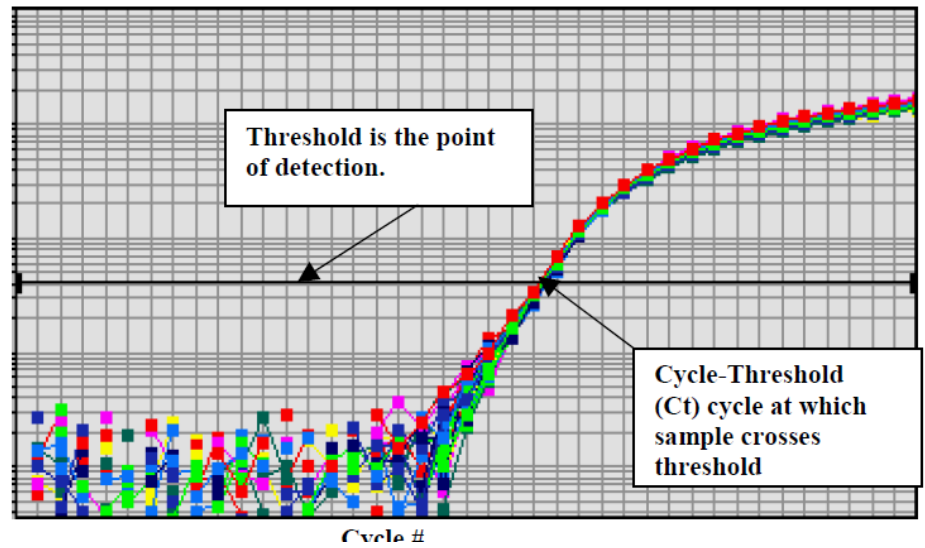
Real time primer design

• 18-24 bases
• 40-60% G/C
• Balanced distribution of G/C and A/T bases
• Avoid runs (≥4) of an identical nucleotide (especially G's)
• Tm that allows annealing at 55-65 °C
• No internal secondary structures (hair-pins)
• The five nt at the 3'end should have no more than two G and/or C bases

Primer pairs should have

• Similar melting temperatures, *Tm ,* within 2-3 °C
• No significant complementarity (> 2-3 bp), particularly not in the 3'-ends

Real Time PCR based Detection of *Ralstonia solanacearum*

Protocol

Reaction Set up

1. Gently vortex and briefly centrifuge all solutions after thawing.
2. Prepare a reaction master mix by adding the following components(except template DNA)
3. Usually the total reaction volume is 25µl.

Reagents	Con. required	volume
Maxima SYBR Green qPCR master Mix (2X)	2X	12.5µl
p759(Forawrd primer)	10pM	1µl
P760(Reverse Primer)	10pM	1µl
Template DNA	200ng	1µl
Nuclease free water		9.5µl

Reaction Conditions

95°C 2min 53C 1min 72C 1min 30sec	1 cycle
94°C 15s 60°C 15sec 72°C 15sec	} 30-35 cycles
72°C 15 min	
8°C forever	
end	

SSR and SNP identification

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SSR identification

Introduction:

Microsatellites, also known as **Simple Sequence Repeats** (SSRs), or sometimes **Short Tandem Repeats** (STRs), are repeating sequences of 1-6 base pairs of DNA. Microsatellites are typically neutral and co-dominant. They are used as molecular markers in genetics, for kinship, population and other studies. They can also be used to study gene duplication or deletion. Microsatellite is a $(CA)_n$ repeat, where n is variable between alleles. The repeated sequence is often simple, consisting of two, three or four nucleotides (di-, tri-, and tetranucleotide repeats respectively), and can be repeated 10 to 100 times.

Objectives:

- To identify the SSRs from Express sequence tag using SSR finding tools.

Exercise 1. Retrieve ESTs from dbEST sequences for SSR identification.

- Go to NCBI site http://www.ncbi.nlm.nih.gov/ select db EST and type either *Phytophthora, Fusarium and Ralstonia* in text box.
- Search will provide either *Phytophthora, Fusarium and Ralstonia* sequences, similar to *Phytophthora* sequences and other.
- Download the EST sequences belongs to *Phytophthora* in fasta format using sent to file or sent to text option.

Exercise 2. Preprocessing (remove poly A/T and vector contamination)

- Go to http://inn.weizmann.ac.il/cgi-bin/EMBOSS/emboss.pl?_action=input&_app=trimest for removing PolyA tail.
- Browse and choose your fasta file or paste your EST sequence.
- Observe the options field manipulate it and click runtrimEST. Immediately output file will open.
- For larger size sequences submit mail ID so that you will get a mail your job is over or not.

Exercise 3. Vector Removal.

- Locate VecScreen server using google or use this direct link http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html
- Paste all the sequences of batch wise
- Run the program.
- It will detect suspected vector sequences in our sequence. We must remove those region or sequences. It may leads to false positive

Exercise 4. Assembling the sequence in to contigs and singletons by CAP3

- Go to CAP3 website http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=cap3 and type your Email ID.
- Paste the EST sequences in the text box or upload the file by selecting file option.
- Click the run button.

- CAP3 will make the EST sequence in to contigs and singletons.
- Once analysed, save the contigs file and singleton file.
- These contigs were used for further analysis.

Exercise 5. Detecting SSRs from contigs ESTs and singletons using SSRprimerII

- Go to SSRprimerII website (**http://flora.acpfg.com.au/ssrprimer2/cgi-bin/index**).
- Upload or paste the sequences file (It will detect di, tri, tetra, penta, hexamer repeats).
- Save the result page.
- Which is the abundant type of repeat?
- Report total number of SSR?

Exercise 6. Detecting SSRs from contigs and singletons using websat

- Go to websat website (http://wsmartins.net/websat/).
- Upload or paste the sequences file (It will detect di, tri, tetra, penta, hexamer repeats).
- Save the result page.
- Which is the abundant type of repeat?
- Report total number of SSR?

Exercise 7. Detecting SSRs using MISA- MIcroSAtellite identification tool (MISA) tool – LINUX OS required.

- Go to MISA website http://pgrc.ipk-gatersleben.de/misa/ download **misa.pl** and **misa.ini** and put in a folder
- Open misa.ini file and edit the file to desired no of repeats in each type of repeat. Eg.
- Use singletons and contigs for this exercise.
- Execute the misa.pl perl script.
- In command line type **misa.pl <FASTAfile>**
- Get the result output **<FASTfile>.misa**, **<FASTfile>.statistics** and interpret your results.
- Analysis the result and categories based on the types of SSR (mono, di, tri, tetra)
- Report abundant type of repeats and class.
- Save the result for primer designing exercise.

SNP identification

Introduction:

A **single-nucleotide polymorphism** (**SNP**, pronounced *snip*) is a DNA sequence variation occurring when a single nucleotide — A, T, C, or G — in the genome (or other shared sequence) differs between members of a species or paired chromosomes in an individual. For example, two sequenced DNA fragments from different individuals, AAGC**C**TA to AAGC**T**TA, contain a difference in a single nucleotide. In this case we say that there are two *alleles*: C and T. Almost all common SNPs have only two alleles.

DNA substitution mutations are of two types.

Transitions are interchanges of two-ring **purines** (**A ↔ G**) or of one-ring **pyrimidines** (**C ↔ T**): they therefore involve bases of similar shape.

Transversions are interchanges of **purine for pyrimidine bases**, which therefore involve exchange of **one-ring** and **two-ring** structures.

Indel - Insertion and deletion of one nucleotide

Objectives:

- To identify SNPs from EST sequences using AutoSNP program

Exercise 6. SNP discovery using ESTs

- This part is analyzed only on LINUX OS.
- To Detect SNP and Indels using AUTOSNP we require the input sequence as either .fasta or .ace format
- You can use .ace file created using Exercise 3 for this analysis.
- CAP3 is integrated in AUTOSNP is used for making clusters.
- For execute the program use following commands.
- In command mode just type **./cap3snp.pl –f <your file name.fasta>**
- if it is ace file means type **./cap3snp.pl –a <your file name.ace>**
- Report No of Transistions/transversions/indels.
- Report the frequency of SNPs.

MicroRNA identification from EST resources

Santhosh J Eapen, A.Chandrasekar and Rosana

MicroRNAs (miRNAs) are short ribonucleic acid (RNA) molecules, on average only 22 nucleotides long and are found in all eukaryotic cells. miRNAs are post-transcriptional regulators that bind to complementary sequences on target messenger RNA transcripts (mRNAs), usually resulting in translational repression and gene silencing. The human genome may encode over 1000 miRNAs, which may target about 60% of mammalian genesand are abundant in many human cell types. In plants the miRNA complementarity to its mRNA target is nearly perfect, with no or few mismatched bases.

- This session teaches you:
- To download miRNA's of specific organisms from miRBASE and MicroCosm databases
- To identify the conserved miRNA's present in a specific organism
- Target gene to which the miRNA's of specific organism binds to for gene silencing
- Prediction of structure of miRNA's

Exercise 1.

Method 1. Download *Oryza sativa* microRNA (mature and precursor) sequence from miRBASE

- Go miRBASE database (use google or the direct link is http://www.mirbase.org/index.shtml)
- Click on **browse** link
- It will display species information and no of microRNAs
- Our intension is to download *oryza sativa* microRNA in mature form and precursor form.
- Find *oryza sativa* from the list
- There are 414 microRNAs for *oryza sativa*
- Click on *oryza sativa* it will show the details.
- Scroll down and use select all option
- Select Sequence type as mature sequence and sequence format unaligned
- Click on fetch sequence button

Method 2. Find all the target of *c. elegan* let-7 microRNA using MicroCosm database

- Go to microcosm database. (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/)
- Enter into database using ENTER Button
- Select *Caenorhabditis elegans* from species list.
- Find let-7 microRNA and explore using view button
- Report the number of target of let-7 microRNA.
- Select any gene to view details
- Highlighted rows in the table indicate genes with published known targets

Exercise 2. Identify conserved microRNAs from *Citrus limon* ESTs.

You can skip the steps 4 -8, if you have high quality sequence. It is mandatory if you are using public recourses.

- Go to NCBI site http://www.ncbi.nlm.nih.gov/ select db EST and type *Citrus limon* in text box.
- Download the fasta format file for analysis.
- In display option select fasta for getting the fasta file.
- By selecting file, whole ESTs can be downloaded in a single file.

Exercise 3: Vector Screening

- Go to http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html for removing vector contamination.
- Copy and paste your fasta file in to the text box.
- Click Run Vecscreen button.
- Find the similarities using Vector Blast. If similarities found delete the similar sequence from the fasta file.
- Use the preprocessed file for further analysis.

Exercise 3. 1: TrimEST

- Access http://inn.weizmann.ac.il/cgi-bin/EMBOSS/emboss.pl?_action=input&_app=trimest for removing Poly A tail.
- Browse and choose your fasta file or paste your EST sequence.
- Observe the options field manipulate it and click run trimEST. Immediately output file will open.
- For larger size sequences submit mail ID so that you will get a mail your job is over or not.

Exercise 3. 2: Trimseq - **Trim ambiguous bits off the ends of sequences**
- Go to http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=trimseq.
- Check file option and browse and choose your file click the RUN button to trim the sequence.
- Save the trimseq output file for further analysis.

Exercise 3. 3: Repeat masking

- RepeatMasker - screens DNA sequences in FASTA format against a library of repetitive elements and returns a masked query sequence ready for database searches.
- Go to the website http://repeatmasker.org in services click repeatmasking
- Browse and choose your fasta file or paste your EST sequence.
- Check search engine as wublast and choose the DNA source.
- Result output will be in HTML format.
- This repeat masked file is used for clustering.

Exercise 3. 4: Clustering and Assembling the ESTs

This step is used to remove the redundant EST (duplicate ESTs) and to assemble EST's.

- Go to http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=cap3 and type your Email ID.
- Paste the EST sequences in the text box or upload the file by selecting file option.
- Click the run button.
- CAP3 will make the EST sequence in to contigs and singletons.
- Once analysed, save the contigs file and singleton file. These contigs and singleton were used to further analysis.
- Use this singletons and contigs sequences for finding the microRNAs

Exercise 3. 5: Find all the conserved MicroRNAs from citrus limon ESTs.

- Use singletons and contigs.
- Do BLAST against miRBASE (which is the public repository of all the validated microRNAs)
- For the above use search option of miRBASE.
- You can create a local blast method to find the microRNA from EST and other resources.
- It is efficient and we can compare all the sequence by one analysis.
- To analyse all the sequence you may use the local blast facility created by iisr miRSeek (http://220.227.138.213/mirnablast/mirnablast.php)
- mirSeek is Local blast utility created by using all the publically available microRNAs latest version 14.
- It can search microRNAs from thousands of sequence or from a file containing multiple sequences at a time.

Exercise 3.6: Find the target gene in which detected microRNAs binds.
Method 1.

- Go to psRNATarget webserver.
- Hyperlink of the same is http://bioinfo3.noble.org/psRNATarget/
- The psRNATarget accepts miRNA/ta-siRNA mature sequences and target candidates as inputs. It reports all potential complementary regions between miRNA/ta-siRNA and target sequences using an improved iterative parallel Smith-Waterman algorithm and a weighted scoring schema allowing each mismatch being penalized according to the mismatch type and position in the query small RNA.
- Paste the mature MicroRNA regions.
- Select a transcript/genomic library for target search
- Leave Maximum expectation and Maximum circles as default value.
- Provide email id and run the program.
- Report all targets.

Method 2.

- Go microinspector tool.
- link is http://bioinfo.uni-plovdiv.bg/microinspector/
- Paste the accession no of your gene sequence/EST sequence Or paste the sequence in sequence submission window
- Select organism from the scroll down menu (eg, *Arabidopsis*)
- Past the microRNA in specified box
- Leave temperature and binding energy as default setting.
- Do search
- Result will show the complementary paired sequence from your submission. That is the gene target for particular microRNA

Exercise 3.7: Predict structure of microRNA

- Go to mfold
- Direct link is http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1-2.3.cgi
- Paste the precursor sequence(sequence containing microRNA region)
    - Provides the stem loop structure with thermodynamic details.

- - Gibbs Helmholtz equation
  - $\Delta G = \Delta H - T\Delta S$
  - ΔG = change in Gibbs free energy, ΔH = change in enthalpy, T = absolute temperature, ΔS = change in entropy
- Observer the result.

Multilocus Sequence Typing of bacteria

A. Kumar

Background

Ralstonia solanacearum Yabuuchi (Smith) previously known as *Pseudomonas solanacearum* Smith is the causative agent of bacterial wilt of crop plants that is regarded as one of the important bacterial plant pathogens owing to its aggressiveness, broad host range & wide geographical distribution and long persistence in soil & water associated environments. The pathogen is known to infect over 450 plant species, including many economically important crops including vegetables, spices, herbaceous plants, shrubs, and trees (Wicker *et al*, 2007). The genetic diversity of *Ralstonia solanacearum* is analysed by several phenotypic and genotypic. The phenotypic assay includes biovar characterization which is based on substrate utilization ability of the bacterium, and host range based race determination (Hayward 1964, Buddenhagen 1962). Five biovars and as many races have been found among the global collection of *Ralstonia solanacearum* (Table 1 & 2).

Though popular, these techniques are not enough for deciphering the population biology of the strains across the geographical locations. Besides, these two independent systems of classification are not in agreement with each other. Perhaps these anomalies prompted the researchers to devise finer tools to reveal the "actual diversity" exist in population of *Ralstonia solanacearum*. Fegan and Prior (2005) devised Phylotyping which classifies Ralstonia solanacearum in to four phylotypes which reflects the geographical origin of the strains: phylotype I and II are composed of Asian and American strains, respectively, whereas phylotype III members are African, and phylotype IV isolates are from Indonesia, Japan and Australia.

Table 1. Classification of *Ralstonia solanacearum* into biovars

Physiological Tests	Biovars				
	1	**2**	**3**	**4**	**5**
Utilization of disaccharides					
Cellobiose	-	+	+	-	+
Lactose	-	+	+	-	+
Maltose	-	+	+	-	+
Oxidation of alcohols					
Dulcitol	-	-	+	+	-
Mannitol	-	-	+	+	+
Sorbitol	-	-	+	+	-

Table 2. Races and the corresponding biovars of *Ralstonia solanacearum*

Race	Host Range	Geographic distribution	Biovar
1	Wide	Asia, Australia	3, 4
		Americas	1
2	Banana, other *Musa* spp.	Caribbean, Brazil, Worldwide	1
3	Potato, some other Solanaceae, Geranium; other species	Worldwide except US and Canada	2
4	Ginger (Zingiberaceae members)	Asia	3,4
5	Mulberry	China	5

Molecular tools for genetic diversity analysis: Many techniques based on the electrophoretic mobility of the genomic fragments are in use for the analysis of population structure of *R. solanacearum* isolates worldwide. The genotypic tool based on electrophoretic patter comparison of PCR/restriction digestion generated fragments (to name few, ISSR, RAPD, Rep-PCR) among the strains is the most popular choice in the late 1990's. These techniques which exploits, the random amplified fragments are turned out to be **Non-Portable Tools** due to their inherent non-reproducible nature. Sequence based discrimination of strains such as Multilocus sequence typing (MLST) and Comparative Genome Hybridization (CGH) which uncovers allelic variants in conserved housekeeping and virulence genes is portable across the laboratories. Sequence data can be compared readily between laboratories, such that a typing method based on the sequences of gene fragments from a number of different housekeeping loci. Multilocus sequence typing approach uses sequences of internal gene fragments and assigns different allele numbers to the sequence at each locus, so it will provide unique allelic profile for each isolate called **Sequence Types** (STs). Based on this approach Castillo and Greenberg (2007) had analyzed the evolutionary forces operating on *R. solanacearum* populations using Multilocus Sequence Typing (MLST) including five housekeeping and three virulence-related genes. *R. solanacearum* to be a diverse pathogen, showing high levels of nucleotide polymorphism and a number of unique alleles in the Chromosome and in the Megaplasmid. So far about 27 to 33 STs were identified for the eight genes by MLST based analysis.

Methodology

Isolation of the *R. solanacearum* isolates
Bacterial wilt affected plant samples were collected from field and processed for isolation of bacterium. The thoroughly washed stem cutting of wilted plants were allowed to ooze in a clean glass of water for few minutes and were plated on to CPG agar amended with 2, 3, 5 triphenyl tetrazolium chloride and incubated at 28°C for two to three days. The typical colonies of *R. solanacearum* as indicated by their fluidal appearance with spiral pink centre were purified by repeated streaking on fresh plates.

Preparation of bacterial cells for DNA isolation
A single colony of *R. solanacearum* was inoculated in a broth and incubated for about 24-36 h for isolation of total genomic DNA.

Isolation of genomic DNA from *R. solanacearum*
DNA isolation

1. Density of the bacterial suspension is adjusted to OD1.0 @ 600nm
2. Spin down at 14000 rpm at room temperature for 2 min.
3. The supernatant is discarded and pellet is washed three times with sterile distilled water.
4. To the pellet 550µl of TE buffer+lysozyme is added, mixed well and incubated for 30 min at 37C.
5. After incubation 76µl of 10% SDS+Proteinase K is added.
6. The contents are mixed by flipping the tube and incubated for 15 min at 65C.
7. After incubation 100µl of 5M NaCl is added and mixed the contents by flipping the tube.
8. Then 80µl of CTAB/NaCl is added, mixed and incubated for 10 min at 65C.
9. After incubation 660µl of Chloroform+isoamyl alcohol is added.
10. The contents are mixed by flipping the tube about 30sec.
11. Then centrifuge for 5 min at 14000 rpm at room temperature.

12. After centrifugation the aqueous fraction is carefully transferred to a new 1.5ml tube without touching the white middle layer (interface). This step is repeated twice.
13. Equal volume of isopropanol is added and inverted to mix.
14. Then centrifuge for 15 min at 14000 rpm at room temperature.
15. The supernatant is gently drained and mixed with 0.5ml of 70% ice cold ethanol.
16. Centrifuge for 15min at 14000rpm at room temperature.
17. After this the supernatant is carefully removed and evaporated the remaining ethanol in the laminar flow for about one hour.
18. 25µl of 10:1 TE is added to each tube to dissolve the DNA and the tubes are kept at4C for overnight.
19. The DNA from the two tubes is pooled in one tube, so the total is 50µl and added RNase to remove the contaminating RNA at a concentration of200µg/ml
20. The tubes are incubated for 30min at 30C. Then stored the DNA at-20C.

Quality analysis and quantitation of genomic DNA

1. 5µl of stock DNA is diluted 10 times by adding 45µl of MQ water.
2. The quantity of DNA is measured using a Biophotometer
3. Quality assessed by gel electrophoresis
4. DNA concentration is adjusted to 200ng per ul of water
5. Proceed with PCR amplification of genes

Multilocus Sequence Typing

Various steps involved in the sequence typing are given in the fig 1. Briefly the selected genes are amplified, purified and sequenced. The sequence reads are assembled and compared with the database for assigning the alleles. The combination of the allele numbers is unique for each strain of the bacterium in question. The allele numbers are further compared among the strains in order to decipher the strain migration in the field of molecular epidemiology.

Choice of loci: For the diversity analysis five housekeeping genes, which resides in the chromosome *(pps*A, phosphoenol pyruvate synthase; *gyr*B, DNA gyrase, subunit B; *adk*, adenylate kinase; *gdh*A, glutamate dehydrogenase oxidoreductase; and *gap*A, glyceraldehyde 3-phosphate dehydrogenase oxidoreductase) and three plasmid borne virulence related genes (*hrp*B, regulatory transcription regulator; *fli*C, encoding flagellin protein; and *egl*, endoglucanase precursor) are considered. The details of the genes, its protein and the conserved length are furnished in the Table 3.

Fig. 1. MLST workflow
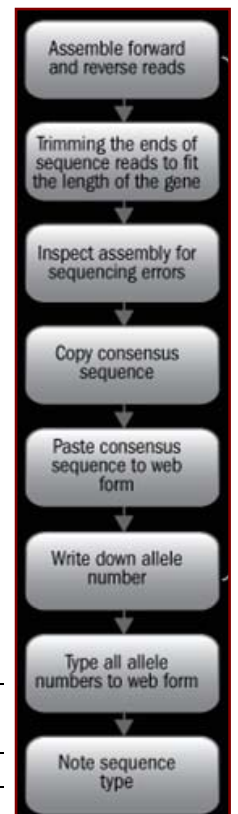
Table 3. Choice of loci for sequence typing of *Ralstonia solanacearum*

Choice of loci	Protein	Length (bp)	*Conserved length (bp)	No of alleles
House keeping genes				
adk	Adenylate kinase	536	420	18
gdhA	Glutamate dehydrogenase oxidoreductase	1000	774	22
gyrB	DNA gyrase subunit B	1710	873	24
gapA	Glyceraldehyde 3-Phosphate dehydrogenase Oxidoreductase	980	774	22

ppsA	Phosphoenol pyruvate synthase	1210	717	23
Virulence related genes				
fli C	Flagellin protein encoding gene	380	318	18
egl	endoglucanase precursor	850	686	24
hrpB	Regulatory transcription regulator	1400	810	25
ppsA	Phosphoenol pyruvate synthase	1210	717	23
		Concatenate sequence length 5.372 kb		

* Needed for final analysis

PCR Amplification: For PCR amplification, the reaction mixture (50μl) contained 50-100ng of template genomic DNA, 1× PCR buffer, MgCl2 3mM, DMSO 6%, each dNTP 50 μM 10pmol of each primer(Table 1), and 1 U of *Taq* DNA polymerase DNA was amplified using an initial denaturation at 96ºC for9 min, followed by 35 cycles of 95ºC for 30s, appropriate annealing for 1 min and extension 72ºC for 2 min. Reactions were completed with a final extension step of 10 min at 72ºC. All PCR products were electrophoresed through a 1.0 % agarose gel and visualized with UV light after ethidium bromide staining.

Elution, Purification and Sequencing: The amplicon was eluted and purified using Gel Elution kit according to the instructions given. The eluted product was sequenced in both directions and the sequences were assembled using DNA baser software. Sequencing is carried out on each DNA strand with BigDye Terminator Ready Reaction Mix under the following conditions, an initial denaturation at 96ºC for 9 min, followed by 35 cycles of 95ºC for 30s, appropriate annealing for 1 min and extension 72ºC for 2 min. Reactions were completed with a final extension step of 10 min at 72ºC. Unincorporated dye terminators were removed by precipitation with 95% alcohol.

Table 2. Primers used in the amplification of gene fragments

Gene	Primer name	Primers for Amplification Sequence(5'-3')	Annealing Temp.	Primers for Sequencing Sequence(5'-3')
gdhA	GdhAF	GATGGATGACGGCCGCATCG	63˚C	GATGGATGACGGCCGCATCG
	GdhAR	TGAACGCCGCCGTCCGCAG		TGAACGCCGCCGTCCGCAG
adk	AdkF	TCTGTTGGGCGCACCCGGC	62˚C	TCTGTTGGGCGCACCCGGC
	AdkR	CCCAGCCGGAGTAGTAGTCC		CCCAGCCGGAGTAGTAGTCC
gyrB	GyrBF	AGGGCTTCGTCGAGTACATCAA	62˚C	AGGGCTTCGTCGAGTACATCAA
	GyrBR	GTTCCGCCGAGGCTCCACG		GTTCCGCCGAGGCTCCACG
gapA	GapAF	ATGACCATCAAGATCGGCAT	55˚C	ATGACCATCAAGATCGGCAT
	GapAR	GGGCCATTTCCAGCACCT		GGGCCATTTCCAGCACCT
ppsA	PpsANF	GGGCGTGATGTTCACGAT	57˚C	GGGCGTGATGTTCACGAT
	PpsANR	CCAGCATGGGGTTCTCTTC		CCAGCATGGGGTTCTCTTC
fliC	Rsol_*fliC*F	GAACGCCAACGGTGCGAACT	63˚C	GAACGCCAACGGTGCGAACT
	Rsol_*fliC*R	GGCGGCCTTCAGGGAGGTC		GGCGGCCTTCAGGGAGGTC
hrpB	RShrpBF	TGCCATGCTGGGAAACATCT	64˚C	TGCCATGCTGGGAAACATCT
	RShrpBR	GGGGGCTTCGTTGAACTGC		GGGGGCTTCGTTGAACTGC

egl	Endo-F	ATGCATGCCGCTGGTCGCCGC	70˚C	ATGCATGCCGCTGGTCGCCGC
	Endo-R	GCGTTGCCCGGCACGAACACC		GCGTTGCCCGGCACGAACACC

Sequence analysis: Sequences are carefully analysed and sequence type assigned for each of the strain by comparing the data sets with www.pamdb.org. The strain relation with the existing collection of strain can be determined by eBurst programme (http://eburst.mlst.net).

Handling sequence data: The sequencing machines would give us the chromatogram indicating the quality of the sequence reads (Fig 2). The sequence reads are carefully observed for any errors in the base using any one of the chromatogram viewers (eg. DNA baser, BioEdit, Chromos etc). Thus obtained sequence is called as raw sequence (Fig.3). For each gene, two such sequences are obtained which are known as forward sequence and reverse sequence respectively. The forward and reverse sequences are assembled using any one of the programmes that are available in public domain (eg. DNA baser). The assemble sequences are called Contigs (large contiguous sequences). Such a long sequences (Contigs) are used to determine the allelic variations (Fig. 4).
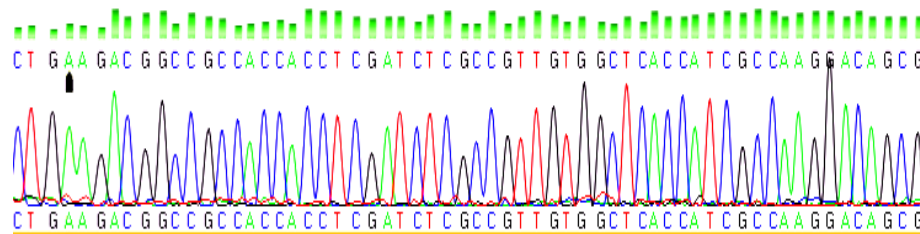
Fig.2. Chromatogram of a sequence read

>A01_CaRS_MepFLIC_FLICF_copy.ab1- Forward Sequence
CTGGGCCAGGTTGAAACAACCTGCAACGTATGCGCCAACTGGCTGTGGAATCCA
ACAACGGCGGTCTGTCGGCAGCCGACCAGACCAACCTGGACAAGGAATACCAAC
AGCTGGCAACGGCTAACAAGAACATCGAAACCAACGCCAACTACAACGGCAACA
AGCTGTTCGACGGCTCGGTGGCTTCGACGACCTTCCAATATGGCCAGAACGCAGC
CACGGACGTGACCACGGTCACCAACGTCAACATGTCGACCTTCGGCACGCTGACC
GGCACGAGCGTGACCAGCGCTGCCAACGCGACCGCAGCCCAGGCCGCGATCGAT
ACCGACCTGACCTCCCTGAAGGCCGCCCA

>A01_CaRS_MepFLIC_FLICR_copy.ab1- Reverse Sequence
GCAGGGCTGCGGTCGCGTTGGCAGCGCTGGTCACGCTCGTGCCGGTCAGCGTGCC
GAAGGTCGACATGTTGACGTTGGTGACCGTGGTCACGTCCGTGGCTGCGTTCTGG
CCATATTGGAAGGTCGTCGAAGCCACCGAGCCGTCGAACAGCTTGTTGCCGTTGT
AGTTGGCGTTGGTTTCGATGTTCTTGTTAGCCGTTGCCAGCTGTTGGTATTCCTTG
TCCAGGTTGGTCTGGTCGGCTGCCGACAGACCGCCGTTGTTGGATTCCACAGCCA
GTTGGCGCATACGTTGCAGGTTGTTTTCAACCTGGCCCAGGTACGAGTCGGCCGT
TTGCAGGTACGAGTTCGCACCGTTGGCGTT

Fig. 3. Fasta file format of forward and reverse sequence reads obtained from sequencing machine

>FliC-CaRs-Mep (393)-Contigs
GGCCTTCAGGGAGGTCAGGTCGGTATCGATCGCGGCCTGGGCTGCGGTCGCGTTG
GCAGCGCTGGTCACGCTCGTGCCGGTCAGCGTGCCGAAGGTCGACATGTTGACGT

TGGTGACCGTGGTCACGTCCGTGGCTGCGTTCTGGCCATATTGGAAGGTCGTCGA
AGCCACCGAGCCGTCGAACAGCTTGTTGCCGTTGTAGTTGGCGTTGGTTTCGATG
TTCTTGTT**A**GCCGTTGCCAGCTGTTGGTATTCCTTGTCCAGGTTGGTCTGGTCGGC
TGCCGACAGACCGCCGTTGTTGGATTCCACAGCCAGTTGGCGCATACGTTGCAGG
TTGTTTTCAACCTGGCCCAGGTACGAGTCGGCCGTTTGCAGGTACGAGTTCGCAC
CGTTGGC

Fig. 4. Assembled sequence for an allele, *fli*C

The assembled sequences are compared with the database (www.pamdb.org) for determination of consensus sequence where the allelic differences can be resolved. The consensus sequence of allele 19 of *Ralstonia solanacearum* is furnished below for illustration (Fig.5).

>CaRs-Mep (318)
GCCGACTCGTACCTGGGCCAGGTTGAAAACAACCTGCAACGTATGCGCCAACTG
GCTGTGGAATCCAACAACGGCGGTCTGTCGGCAGCCGACCAGACCAACCTGGAC
AAGGAATACCAACAGCTGGCAACGGCTAACAAGAACATCGAAACCAACGCCAAC
TACAACGGCAACAAGCTGTTCGACGGCTCGGTGGCTTCGACGACCTTCCAATATG
GCCAGAACGCAGCCACGGACGTGACCACGGTCACCAACGTCAACATGTCGACCT
TCGGCACGCTGACCGGCACGAGCGTGACCAGCGCTGCCAACGCGACC

Fig.5. Allele 19 of gene *fli*C belong to *Ralstonia solanacearum* infecting Zingiberaceae members

The string of allele numbers (integers) for the housekeeping and virulence genes obtained for a strain is called as sequence type which is specific for a strain of bacterium. For example, the allele numbers obtained for a cardamom strain of *Ralstonia solanacearum* is *pps*A-10, *fli*C-19, *hrp*B-27, *gdh*A- 24, *adk*-1, *gyr*B-26, *egl*-25. The combination of integers (10, 19, 27, 24, 1, 26, and 25) serves the input data for establishing the strain relationship by eBurst programme which is based on eBurst algorithm, a dedicated programme for analysis of microbial MLST data.

Phylogenetic analysis using MLST data
The allelic sequences, thus, obtained from the strains are pooled to construct concatenated sequences which serve input data for establishing phylogeny. The concatenated sequence is nothing but the string of all the loci are assembled in an order (**ppsA + fliC + hrpB + gdhA + adk + gyrB + egl**) to get large sequence length. An example of concatenated sequence constructed for a strain of *Ralstonia solanacearum* obtained from cardamom is furnished below (Table 3, Fig.6). This large sequence length is used in the phylogenetic analysis of bacterium in question.

>*R_solanacearum__*CaRs-Mep_ (911) [ppsA + fliC + hrpB + gdhA + adk + gyrB + egl]
GACGAAGACGTGGTCGAGCTGGCCAAGTACGCCGTCATCATCGAGAAGCACTAC
GGTCGCCCGATGGACATCGAGTGGGGTAAGGACGGCAAGGACGGCAAGATCTAC
ATCCTGCAGGCCCGCCCCGAGACGGTGAAGAGCCAGTCGGTCGGCAAGGTCGAG
CAGCGCTTCCGCCTGAAGGGCTCGGCGCCGGTGCTGACCACCGGCCGCGCGATCG
GCCAGAAGATCGGTACGGGCCCCGTGCGCGTGATCAACGATCCGGCCGAAATGG
AGCGCGTGCAGCCGGGCGACGTGCTGGTCGCCGACATGACCGACCCGAACTGGG
AGCCGGTGATGAAGCGCGCCTCGGCCATCGTCACCAACCGTGGCGGCCGCACCT
GTCACGCCGCCATCATCGCGCGTGAGCTGGGCGTGCCGGCCGTGGTCGGCTGCGG
CGACGCCACCGACCTGCTGAAGGACGGCACGCTGGTCACCGTGTCCTGCGCCGA
GGGCGACGAAGGCAAGATCTACGACGGCCTGCTCGAGACGGAAATCACCGAAGT

GCGCCGCGGCGAGATGCCGCCGATCGACGTCAAGATCATGATGAACGTCGGCAA
CCCGCAGCTGGCCTTCGAGTTCGCGCAGATCCCGAACGGCGGCGTGGGCCTGGCC
CGCCTCGAGTTCATCATCAACAACAACATCGGCGTCCACCCGAAGGCGATCCTCG
ACTACCCGCAAGCCGACTCGTACCTGGGCCAGGTTGAAAACAACCTGCAACGTAT
GCGCCAACTGGCTGTGGAATCCAACAACGGCGGTCTGTCGGCAGCCGACCAGAC
CAACCTGGACAAGGAATACCAACAGCTGGCAACGGCTAACAAGAACATCGAAAC
CAACGCCAACTACAACGGCAACAAGCTGTTCGACGGCTCGGTGGCTTCGACGAC
CTTCCAATATGGCCAGAACGCAGCCACGGACGTGACCACGGTCACCAACGTCAA
CATGTCGACCTTCGGCACGCTGACCGGCACGAGCGTGACCAGCGCTGCCAACGC
GACCGTGCTGGCGATGGCCGATGCCTCGCTGCTGCTCGAGTGCGATGAAGAAGC
GGAAGAAGGCTTCCGCCTGGCGCAGCGCCTGATCCGCCATTCGGATGACCAGCTG
CGCGTGGTGTCGTGCCGCAATACCGGCTGGCAGGCACTGCTGCGCGATCGCTACG
CCGCGGCGGCGAGCTGCTTCTCGCGCATGGCCGAAGACGATGGCGCGACCTGGA
CCCAGCAGGTCGAGGGCCTGATCGGCCTGGCGCTGGTGCATCACCAGCTCGGCCA
GCAGGATGCCTCCGACGACGCGCTGCGGGCGGCGCGCGAGGCCGCAGACGGCCG
CAGCGATCGCGGCTGGCTGGCCACCATCGATCTGATCATCTACGAATTCGCCGTG
CAGGCCGGCATCCGCTGCTCCAACCGCCTGCTCGAGCATGCGTTCTGGCAATCGG
CCGAAATGGGCGCGACCCTGCTGGCCAACCACGGCGGCCGCAACGGTTGGACGC
CGACCGTATCGCAGGGCGTACCGATGCCGGCGCTGATCCAGCGCCGCGCCGAAT
ACCTCAGCCTGCTGCGCCGCATGGCCGACGGGGACCGCGCGGCAATCGACCCGC
TGATGGCGACCCTCAACCACTCGCGCAAGCTCGGCAGCCGCCTGCTGATGCAGAC
CAAGGTGGAAGTCGTGCTGGCCGCGCTGAGCGGCGAGCAGTACGACGTCGCCGG
CCGCGTCTTCGACCAGATCTGCAACCGCGAGACCACCTACCGCGCGCGCCGCTGG
AATTTCGACTTCCTCTACTGCCGCGCCAAGATGGCCGCCCAGCGCGGCGACTCGG
TCAAGAACGCGGCCGTCAACGTGCCGTACGGCGGCGCCAAGGGCGGCGTCCGCG
TCGATCCGCGCAAGCTGTCGTCGGGCGAACTCGAGCGCCTGACCCGCCGCTACAC
CAGCGAGATCGGCATCATCATCGGCCCGAACAAGGACATCCCGGCGCCGGACGT
GAACACCAACGCGCAGATCATGGCGTGGATGATGGACACGTACTCCATGAACGA
AGGCGCCACCGCCACCGGCGTGGTGACCGGCAAGCCGATCGCGCTGGGCGGCAG
CCTGGGCCGCCGCGAGGCGACCGGCCGCGGCGTGTTCGTGGTCGGCAGCGAGGC
TGCACGCAATCTGGGCATCGACGTCAAGGGTGCGCGCATCGTGGTGCAAGGCTTC
GGCAACGTCGGCAGCGTGGCCGCCAAGCTGTTCCAGGATGCCGGCGCCAAGGTG
ATCGCGGTGCAGGACCACAAGGGCATCGTGTTCAACGGCGCGGGCCTGGACGTC
GACGCGCTGATCCAGCACGTGGACCATAACGGCAGCGTCGACGGCTTCAAGGCC
GAGACCCTGTCGGCGGACGATTTCTGGGCGCTGGAATGCGAATTCCTGATCCCGG
CCGCGCTCGAAGGCCAGATCACCGGCAAGAACGCGCCCCAAATCAAGGCAAAAA
TTGTCGTTGAAGGTGCAAACGGCCCCACGACGCCCGAAGCGGACGACATCCTGC
GCGATCGCGGCATCCTGGTCTGCCCGGACGTGATCGCCAATGCCGGCGGCGTCAC
GGTGAGCTATTTCGGCATTCCGCAGATCTCCACCGGCGACATGCTGCGCGCCGCC
GTCAAGGCCGGCACCCCGCTGGGCATCGAAGCCAAGAAGGTGATGGACGCCGGC
GGCCTGGTGTCCGACGACATCATCATCGGCCTGGTGAAGGACCGCCTGCAGCAGT
CCGACTGCAAGAACGGCTACCTGTTCGACGGCTTCCCGCGCACCATCCCCCAGGC
CGAAGCCATGAAGGATGCCGGCGTGCCGATCGACTACGTGCTGGAAATCGACGT
GCCGTTCGACGCCATCATCGAGCGCATGAGCGGCCGCCGCGTGCACGTGGCCTCG
GGCCGGACCTATCACGTCAAGTACAACCCGCCCAAGAACGAGGGCCAGGACGAC
GAAACCGGCGATCCGCTGATCCAGCGCGACGACGACAAGGAAGAAACCCCTGAC
CGGCCTGCGCGCCGCGATGACGCGCGTCATCAACAAGTACATCGCCGACAACGA
GATCGCCAAGAAGGCCAAGGTCGAAACCTCCGGCGACGACATGCGCGAAGGCCT
GACCTGCGTGCTGTCGGTGAAGGTGCCCGAGCCCAAGTTCAGCTCGCAGACCAA
GGACAAGCTCGTTTCGTCCGAAGTGCGCCTGCCGGTGGAAGAAGTCGTGGCCAA
GGCGCTGACGGACTTCCTGCTGGAGACGCCCAACGACGCCAAGATCATCTGCGG
CAAGATCGTTGAAGCCGCGCGTGCCCGCGAAGCCGCCCGCAAGGCCCGCGAGAT
GACGCGCCGCAAGGGCGTGCTCGACGGCATGGGCCTGCCCGGCAAGCTGGCCGA
CTGCCAGGAGAAAGACCCGGCACTGTCCGAACTGTTCATCGTCGAGGGTGACTCC

GCAGGCGGCTCGGCCAAGCAGGGCCGCGACCGCAAGTTCCAGGCGATCCTGCCG
CTCAAGGGCAAGATCCTGAACGTGGAGCGCGCGCGCTTCGACAAGATGCTCTCC
AGCCAGGAAGTGCTCACGCTCATCACCGCCATGGGCACCGGCATCGGCAAGGAC
GACTACAACCTCGACAAGCTGCGCTATCACCGCATCATCATCATGACCGACGCGG
ACGTGGACGGCTCGCACATCCGCACGCTGCTGCTGACGTTCTTCTACCGCCAGAT
GCCCGAGATCATCGAGCGCGGCCACGTGTACATCGCCCAGCCGCCGCTGTACAA
GATCAAGCACGGCAAGGAAGAGCGCTACATCAAGGACGACAACGAGATGGCCG
CGTACCTGATGCGCCAGGCGCTCGACACCGCCATCCTGGTGCGCGCCGACGGCAC
CACCCTCAGTACGGCGGCCGCTACCGACACCACGACCCTGAAGACGGCCGCCAC
CACCTCGATCTCGCCGTTGTGGCTCACCATCGCCAAGGACAGCGCGGCGTTCACG
GTGAGCGGCACGCGCACGGTGCGCTATGGCGCCGGCAGCGCGTGGGTGGCGAAG
AGCATGTCCGGCACAGGCCAGTGCACCGCCGCCTTCTTTGGCAAGGATCCGGCGG
CCGGTGTCGCCAAGGTATGCCAGGTGGCGCAGGGCACGGGCACCCTGCTGTGGC
GCGGCGTCAGCCTGGCCGGCGCCGAGTTCGGGGAGGGCAGCCTGCCGGGCACCT
ACGGGAGCAACTACATCTATCCGTCCGCCGACAGCGCGACCTACTACAAGAACA
AGGGCATGAACCTCGTGCGCCTGCCGTTCCGCTGGGAGCGGCTGCAGCCCACGCT
CAACCAGGCGCTCGACGCGAACGAGCTGTCGCGCCTGACCGGGTTCGTCAACGC
CGTGACGGCGGCCGGCCAGACGGTGCTGCTCGATCCGCACAACTACGCGCGCTA
CTACGGCAACGTGATCGGCTCGAGCGCGGGTGCCCAACAGCGCGTACGCCGATTTC
TGGCGGCGCGTGGCCACCCAGTTCAAGGGCAATGCCCGCGTCATCTTCGGGCTGA
TGAACGAGCCCAATTCGATGCCGACCGAGCAGTGG

Fig.6. Concatenated sequence obtained for a strain of *Ralstonia solanacearum*

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Transcriptomics

Johnson K. George

Introduction

The transcriptome is the complete set of transcripts in a cell, and their quantity, for a specific developmental stage or physiological condition. Understanding the transcriptome is essential for interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues, and also for understanding development and disease. The key aims of transcriptomics are: to catalogue all species of transcript, including mRNAs, non-coding RNAs and small RNAs; to determine the transcriptional structure of genes, in terms of their start sites, 5′ and 3′ ends, splicing patterns and other post-transcriptional modifications; and to quantify the changing expression levels of each transcript during development and under different conditions.

Traditional sequencing approaches require cloning of DNA fragments into bacterial vectors for amplification and sequencing of individual templates using vector-based primers. This approach was adapted for cDNA libraries and, with the advent of capillary sequencing, became suitable for high-throughput sequencing of large samples of transcripts, termed Expressed Sequence Tags (ESTs). ESTs have become an invaluable resource for gene discovery, functional genomics (genome annotation), alternative splicing, SNP discovery, molecular markers for population analysis, and expression analysis in animal, plant, and microbial species.

However, large-scale EST sequencing using the traditional Sanger method is time consuming and expensive. During the past several years, the NGS technology has become a tremendous approach for high-throughput gene discovery on a genome-wide scale in different organisms.

The newly developed high throughput sequencing technology, i.e. Next Generation Sequencing (NGS), including the Roche/454 Genome Sequencer FLX Instrument, the ABI SOLiD System, and the Illumina Genome Analyser, is a powerful and cost-efficient tool for advanced research in many areas, including re-sequencing, micro- RNA expression profiling, DNA methylation, especially de novo transcriptome sequencing for different organisms.

Over the past years, NGS has greatly accelerated our understanding of the complexity of gene expression, regulation and networks in model and non-model organisms. Though the transcriptome sequencing for non-model organisms using NGS was almost confined to 454 pyrosequencing due to its longer read length compared with the other two platforms, it is noteworthy that a draft genome sequence for the giant panda has been generated and assembled successfully using only Illumina Genome Analyser sequencing technology. Recently whitefly transcriptome was also characterized using this short read sequencing platform.

Sequencing platforms

At present, three platforms dominate the NGS market. The FLX system from 454 (a Roche company, Basel, Switzerland), the SOLiD system from ABI, Foster City, California, USA and the Genome Analyser commercialized by Illumina, San Diego, California, USA. The protocol presented here is designed for the Illumina Genome Analyser, which is currently the most widely available platform http://www.illumina.com/technology/sequencing_technology.ilmn). This platform allows the

parallel analysis of millions of DNA molecules producing short sequence reads of 37 to over 70 nucleotides in length, depending on the sequencing conditions and machine version used. To achieve this, a library of short DNA molecules with specific adaptors ligated at both ends is applied to a sealed glass device called a flow cell. The surface of the flow cell is coated with oligonucleotides specific for the library adaptors, allowing single DNA molecules to bind randomly to its surface. In a second step, each isolated DNA molecule is amplified locally on the slide (bridge amplification), creating distinct clusters of identical DNA molecules. These clusters are then sequenced by synthesis, in parallel, on the surface of the flow cell. For each round of sequencing, the four nucleotides coupled to four different fluorophores are added at the same time to the flow cell. Analogous to the use of standard chain terminator ddNTPs, the fluorophore-tagged dNTPs are reversibly chemically blocked to ensure that only a single incorporation occurs. After each round of dNTP incorporation, the slide is then imaged to record the nucleotide that has been added to each cluster. Blocking groups and fluorophores are finally removed before continuing with the next round. Each run produces terabytes of image data that are analyzed for the fluorescence emitted by each cluster at each cycle, thus allowing deduction of the cluster DNA sequence.
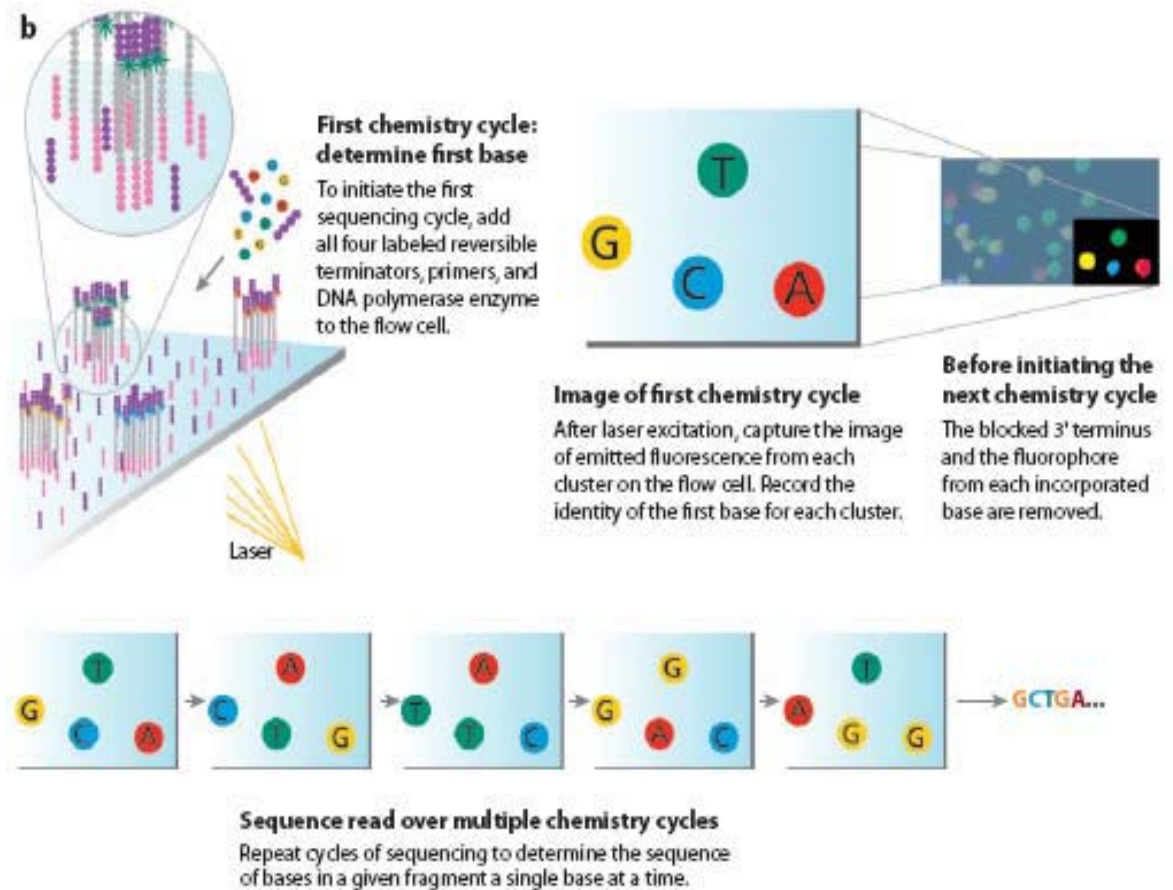
Transcriptome sequencing

To maximize the diversity of sequences retrieved from RNA-seq libraries, it is therefore advisable to reduce the quantity of rRNA present in the sample. This can be achieved either by enrichment for polyadenylated RNA or by depletion of rRNA species. Because of the huge depth of sequencing data generated by NGS, small amounts of contaminating genomic DNA may appear to be transcribed RNA when the sequencing results are analyzed. It is therefore important to treat the enriched RNA samples with DNase to completely remove any remaining amounts of DNA. After a column purification (Qiagen, Hilden, Germany) of the DNase digests, the enriched RNA samples are then converted to cDNA using poly(dT) or random primers. Although priming reverse transcription with poly(dT) further enriches the library for sequences derived from poly(A) + RNA, it also reduces the representation of the 5′ ends of long transcripts. The use of random primers represents a good alternative, ensuring a more uniform sequence representation across transcripts. Finally, second-strand cDNA synthesis is carried out to obtain double-stranded cDNA, which can in turn be processed using the Illumina library creation protocol originally developed for genomic DNA. In this procedure, the cDNA sample is first fragmented into pieces of a size compatible with downstream cluster formation and sequencing. This is usually carried out by nebulization, but other techniques have been explored. Nebulization (or other fragmentation methods) generates double-stranded cDNA fragments with a mix of blunt ends as well as 3′ overhangs. The library needs, therefore, to be treated with Klenow and T4 DNA polymerases to obtain fragments with uniform blunt ends. Next, adenine residues are added to the fragment extremities using the Klenow enzyme to prepare the library for efficient adaptor ligation. Finally, the DNA adaptors necessary for cluster formation, amplification and sequencing are ligated at both ends of the fragments using a T4 DNA ligase. After PCR amplification of the library for a limited number of cycles, fragments of 120–170 bp in length are size-selected on an agarose gel. This step will remove unincorporated adaptors and ensure that the average fragment size of the library is optimal for attachment to the flowcell and cluster formation. Such RNA-seq libraries are ready for cluster formation and loading on the Genome Analyser.
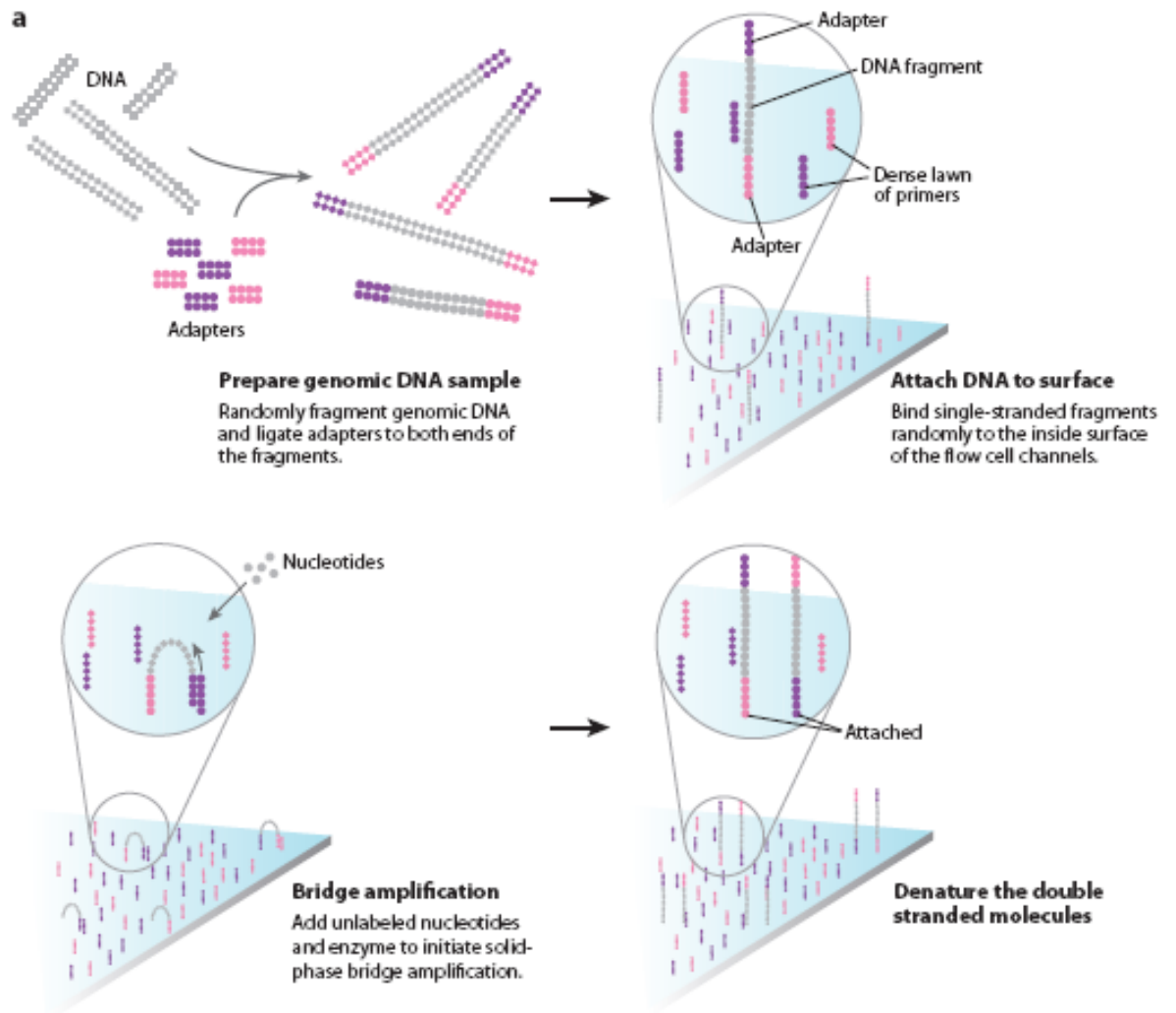
A) Bridge amplification.

Alternative experimental design described here has the advantage of being robust and of being partially based on the genomic DNA library creation protocol initially developed by Illumina. These features have the advantage that most new technological developments optimized for genomic DNA sequencing should be easily transferable to this protocol and, therefore, to the analysis of cDNA. Paired-end analysis, for instance, is a variation of the classical Illumina sequencing protocol, where both ends of each library fragment are sequenced11. Applied to RNA-seq, this approach could help in deciphering the structure of complex transcriptomes rich in repetitive sequences. Another variation, called multiplexing, allows sequencing of pools of samples in a single reaction (each library being tagged with a different DNA sequence index). This approach permits reduction of the amount of resources needed per sample at the cost of sequence depth. It could be an attractive option for small transcriptomes.

b

First chemistry cycle: determine first base

To initiate the first sequencing cycle, add all four labeled reversible terminators, primers, and DNA polymerase enzyme to the flow cell.

Laser

Image of first chemistry cycle

After laser excitation, capture the image of emitted fluorescence from each cluster on the flow cell. Record the identity of the first base for each cluster.

Before initiating the next chemistry cycle

The blocked 3' terminus and the fluorophore from each incorporated base are removed.

GCTGA...

Sequence read over multiple chemistry cycles

Repeat cycles of sequencing to determine the sequence of bases in a given fragment a single base at a time.

B) Sequencing by synthesis

Conclusion

Genomics-assisted breeding approaches have greatly advanced with the increasing availability of genome and transcriptome sequence data for several model plant and crop species. The development of second generation sequencing technologies and beyond, provide opportunities to develop millions of novel markers, in non-model crop species as well, for identification of genes of agronomic importance. Identification of all genes within a species permits an understanding of how important agronomic traits are controlled, knowledge of which can be directly translated into crop improvement.

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Gene silencing using RNAi approaches

Debasis Pattanayak

Introduction

RNA-mediated gene silencing in plants is a collective manifestation of broadly two different functions- defense against molecular parasites like viruses, transposons and introduced transgenes by either post-transcriptional or transcriptional gene silencing, and orchestration of developmental stages by regulation of endogenous gene expression at post-transcriptional level. RNA silencing has pervaded our scientific knowledge on gene regulation and has made great impact in deciphering gene functionality and crop improvement.

Small RNAs as sentinels for plant defense

Plants are continuously being invaded by pests and diseases. Among these invaders molecular parasites like viruses, transposons and transgenes are of special importance as they either capture or direct cellular machinery for their proliferation, or cause genome instability through genome reorganization. In response, plants have evolved an equally efficient defense strategy to counter attack invasion of molecular parasites mediated by small interfering RNA (siRNA) of 21-24 nt long. siRNAs are generated in response to long dsRNA and induce target gene silencing either at post-transcriptional or transcriptional level.

Post-transcriptional ablation of mRNA of molecular invaders like viruses is mediated by 21 nt siRNA, and the process has been termed as post-transcriptional gene silencing (PTGS) in plants. The basic process of PTGS involves cleavage of long dsRNA into siRNAs, which guide degradation of complementary target mRNA. Replicating intermediates of plant RNA viruses, unintended transcription of nuclear gene by neighbouring promoter(s), engineered inverted repeat transgene or exogenously introduced synthetic dsRNA are the probable source of generation of silencing trigger of dsRNA in plant. When encountered with the long dsRNA an endoribonuclease called Dicer progressively cleaves the precursor RNA into ~21 nt long siRNA. In *Arabidopsis* four Dicers called Dicer-Like 1-4 (DCL1-4) are present and probably DCL2 and DCL4 are involved in generation of siRNAs involved in PTGS. Double stranded siRNAs then get incorporated into another multiprotein ribonuclease complex, RISC (RNA-induced silencing complex). RISC-siRNA assembly undergoes ATP-dependent activation step of unwinding of double-stranded siRNA to single-stranded siRNA. Activated RISC then targets and cleaves mRNA guided by sequence complementarity with siRNA. Cleaved mRNA eventually becomes target for further degradation.

Gene transcripts that do not have dsRNA intermediate but sensed by the cell as aberrant or foreign such as highly expressed sense transgene mRNAs and plant DNA viruses are used as template by cellular RNA-dependent RNA polymerase (RdRp) to synthesize dsRNA intermediate without the aid of primer. This dsRNA intermediate is then cleaved by DCL2/DCL4 and channeled into normal route of PTGS.

Plant defense through siRNA-mediated transcriptional silencing of target gene has been manifested by DNA methylation and histone modification. This defense mechanism is particularly directed to silence transposons and endogenous genes having repeat elements. The peculiarity of RNA-mediated transcriptional silencing is persistence of silencing signal even in the absence of silencing trigger. This is ensured by the epigenetic mark of DNA and histone hypermethylation and heterochromatinization of corresponding gene loci. Like PTGS, silencing has been initiated by Dicer cleavage of dsRNA generated from the transcription of inverted repeats or by the action of RdRp termed RDR2 in *Arabidopsis* on single-stranded template. However, unlike PTGS, a different Dicer, DCL3 in *Arabidopsis*, generates a

different size class of siRNAs of 24 nt long through endonucleolytic cleavage of dsRNA. The silencing initiator complex then recruits different DNA methyl transferases like domain rearranged methyltransferases (DRM) and methyltransferase 1 (MET1) for target DNA methylation guided by siRNA sequence. Methylated DNA sequence is probably recognized by methyl cytosine binding protein (MBD), which then recruits histone deacetylase for deacetylation of lysine-9 residue of histone 3 (H3K9) and histone methylase (KRYOTONITE) for methylation of H3K9. These epigenetic marks cause transcriptional silencing and maintenance of silent state is ensured by the involvement of other protein factors.

Small RNAs as developmental regulators

Another class of small non-coding 21nt-long RNAs is involved in plant developmental fate determination by negatively regulating expression of genes encoding transcription factors that orchestrate developmental differentiation. This class of small RNA called microRNA (miRNA) originates from intergenic region, introns or even coding region of genes most probably by the action of RNA polymerase II. Transcription of miRNA encoding genes, *MIR*, produce long stem loop primary transcript termed pri-miRNA. Primary transcript, pri-miRNA, has been processed in the plant nucleus by Dicer, DCL1, into duplex miRNA through an intermediate hairpin-stem structure called pre-miRNA. Either the duplex miRNA or mature single-stranded miRNA is exported into cytoplasm mediated by plant protein HASTY. In the cytoplasm mature miRNA gets incorporated into a ribonucleoprotein complex, miRNP, which is similar to RISC. miRNP-miRNA assembly prevent target gene expression either by cleavage of homologous mRNA similar to PTGS or translational repression after binding to homologous mRNA at the 3′ untranslated region. To date a number of plant miRNAs have been identified either by computational prediction or by experimental validation. Sequence of most of the plant miRNAs has been found to be conserved among related organisms. Majority of so far identified plant miRNAs target transcription factor genes with perfect sequence complementarity and elicit transcript cleavage. *Arabidopsis* miRNAs, miR165/166 that target PHABULOSA/ PHABOLUTA/ REVOLUTA transcription factor genes and regulate leaf polarity; miR-JAW that target TCP transcription factor gene (CINCINNATA) and regulate leaf development, belong to this class. To date only exception to this generalization of plant miRNA function has been observed with plant miRNA 172 regulating target gene expression by translational repression. Genes encoding a group of floral developmental repressor transcription factors, APETALA2, are targets of miR172 in *Arabidopsis*.

RNAi in crop improvement

Various approaches have been used to produce dsRNA in plants. Initially this was achieved by transforming plants with constructs to produce sense and antisense RNA. A more amenable approach has been using both sense and anti-sense sequences, separated by an intron. Upon transcription, these sequences form a hairpin RNA (hpRNA) molecule that triggers gene silencing. This technology of expression of hairpin constructs has been applied to a wide range of species to silence the expression of both endogenous genes and genes of invading pathogens. The first convincing evidence of specific and heritable dsRNA-mediated gene silencing was in *Arabidopsis*. Introduction of inverted repeat gene construct against gene involved in floral development created both reduction and loss of function mutants. Since then innumerable reports of metabolic engineering of plants using RNAi have been reported. Silencing of two key enzymes in the fatty acid biosynthesis pathway in cotton, ghSAD-1 and gh-FAD2-1, resulted elevation of stearic acid and oleic acid content, respectively, in transgenic lines. Low level of accumulation of theobromine and caffeine was obtained by manipulation of caffeine biosynthetic pathway in coffee using RNAi. This was a major breakthrough towards development of engineered decaffeinated coffee plants. Similarly, silencing of codeinone reductase (COR) gene in opium poppy resulted in development of nonnarcotic alkaloid containing transgenic poppy lines. This was the first report of metabolic

engineering of the opium poppy using RNAi to interfere with multiple steps in a complex biosynthetic pathway. Improvement in productivity of oilseed rape was achieved using an hpRNA construct targeted against MADS-box floral organ identity genes, *BP1* gene family, under the control of a chimeric petal-specific promoter derived from *Arabidopsis*. The resultant plants produced male fertile flowers in which the petals were converted into sepaloid petals. Increase in carotenoid and flavonoid content in tomato was obtained by silencing of an endogenous photomorphogenesis regulatory gene, *DET1*, using a fruit-specific promoter. In transgenic lines all other parameters for fruit quality remained unchanged except for increase in carotenoid and flavonoid content.

As RNAi is the natural defense mechanism against invading pathogens, especially viruses, many attempts have been made for development of virus resistant transgenic plants with mixed success. One of the breakthrough achievements of use of RNAi in development of disease resistant transgenic plants is engineering root-knot resistance in transgenic *Arabidopsis* by RNAi silencing of a nematode parasitism gene. Root-knot nematode parasitism gene *16D10* encodes a conserved secretory peptide that stimulates root growth and functions as a ligand for a putative plant transcription factor. Resistance against root-knot nematode has been achieved through *in vitro* and *in vivo* RNAi approaches to silence *16D10* gene. Ingestion of 16D10 dsRNA *in vitro* silenced the target parasitism gene in root-knot nematode and resulted in reduced nematode infectivity. *In vivo* expression of 16D10 dsRNA resulted in resistance effective against the four root-knot nematode species.

It has been shown recently that both animal and plant miRNA precursors can be modified to express a small RNA with a sequence that is unrelated to the miRNA normally produced by the precursors. Based on *in vitro* analysis and study of plant miRNAs and their targets, specific sequence parameters important for target selection by plant miRNAs have been proposed. Using these determinants artificial miRNAs (amiRNAs) were designed to target individual genes or groups of endogenous genes in *Arabidopsis*. These miRNAs were found to be highly specific in silencing targeted genes without any off-target effect. amiRNA, when put into the backbone of endogenous pre-miRNA, was processed normally and produced phenocopies of multiple mutant combinations of genes that are not naturally regulated by miRNA. In addition *Arabidopsis* based synthetic pre-miRNAs were found to be processed effectively in tomato and tobacco. Real breakthrough in amiRNA-mediated gene silencing was achieved through development of turnip yellow mosaic virus (TYMV) and turnip mosaic virus (TuMV) resistant transgenic *Arabidopsis* by overexpression amiRNA targeted against the viral genes.

RNAi-mediated gene silencing for improvement of potato chip colour: case study
Cold storage of potato is an integral requirement for post-harvest handling of this semi-perishable crop. Unfortunately, potato accumulates high amount of reducing sugars during cold-storage, an unwanted physiological phenomenon known as cold-induced sweetening (CIS). Processed products like chips, French fries etc., when prepared from sweetened potatoes, develop brown colouration, and are not preferred for processing and table purposes alike. None of the so far bred Indian potato cultivars are good cold-chippers, and this poses a serious impediment to otherwise fast emerging potato processing industry in India because of lack of supply of quality raw material round the year. One rate limiting steps towards reducing sugars accumulation in cold stored potatoes is hydrolysis of sucrose into glucose and fructose by the vacuolar enzyme acid invertase (INV). Improvement of cold-chipping attributes has been achieved by RNAi -mediated suppression of UDP-glucose pyrophosphorylase (UGPase*)* and vacuolar acid invertase (INV*)* genes employing three different approaches.

Internodal stem explants of Indian potato cultivar, Kufri Chipsona-1, were transformed using *Agrobacterium tumefaciens*, and the putative transformants were screened for *NPTII*

expression by RT-PCR. Five transgenic lines, selected after repetitive glasshouse trials based on yield, cold-chipping performance and reducing sugars level after cold storage at 2 °C for three months, were evaluated under confined field condition. Yield of two transgenic events (K.ChipInvRNAi-2214 & -2123) were found comparable to non-transgenic wild type control, Kufri Chipsona-1. Although, only one invertase RNAi transgenic event, K.ChipInvRNAi-2214, produced acceptable chip colour when evaluated directly after 45 days of cold storage, five transgenic events produced acceptable chip colour after 21 days of reconditioning. Similar pattern in chip colour was obtained after 90 days of cold storage. Highest level of reduction of reducing sugars (glucose + fructose) level was observed in K.ChipInvRNAi-2214 at all the stages of cold storage. Reducing sugars level in K.ChipInvRNAi-2214 was either below or equal to the acceptable limit of 100 mg/100gFW. Reduction of reducing sugars level in K.ChipInvRNAi-2214, was 91% directly after three months of cold storage, while it was 82% after 21 days of reconditioning.

The Southern analysis revealed that K.ChipInvRNAi-2214 carried single copy of the transgene, while other lines had two or three copies of the transgene. Reduction in *INV* expression in the five RNAi transgenic events was determined by Northern analysis. Steady state *INV* mRNA level declined substantially in the transgenic lines compared to that of wild type control, and the highest level of reduction was observed in the K.ChipInvRNAi-2214. *INV* specific siRNA accumulation was observed in all the transgenic lines, and the siRNA level correlated well with decline in *INV* transcript.

Apart from siRNA-mediated silencing of potato vacuolar acid invertase gene at the post-transcriptional level, two other different approaches for reduction of cold-induced sweetening in potato have also been attempted - artificial microRNA (amiRNA)-mediated silencing of potato UDP-glucose pyrophosphorylase (*UGPase*) and vacuolar invertase (*INV*) genes, and transcriptional silencing of potato *UGPase* gene. A total of eight amiRNAs, four against *UGPase* mRNA and four against *INV* mRNA, targeting different regions of the target mRNA but without any no off-target effects, were selected using web-based bioinformatics tool. *UGPase* amiRNAs were put into *Arabidopsis* pre-miRNA 164b stem-loop backbone, and *INV* amiRNAs were put into *Arabidopsis* pre-miRNA 164a stem-loop backbone. The pre-miRNA backbones harbouring different amiRNAs were put under the control of endogenous potato *UGPase* promoter and *NOS* terminator. *UGPase* transcriptional gene silencing construct (iIR-*UGPase*-TGS) was developed by putting inverted repeat *UGPase* promoter intervened by *UGPase* intron under the control of potato GBSS promoter.

A total of 179 transgenic lines (31 for *UGPase* amiRNA, 87 for *INV* amiRNA and 23 for both *UGPase* and *INV* combined amiRNAs, 38 *UGPase*-TGS) of three Indian potato cultivars, Kufri Chipsona-1, Kufri Chipsona-3 and Kufri Surya was evaluated under glasshouse, and 50 transgenic lines (13 for *UGPase* amiRNA, 14 for *INV* amiRNA and 11 for both *UGPase* and *INV* combined amiRNAs, 12 *UGPase*-TGS) possessing acceptable chip colour at fresh harvest were selected. Seven transgenic lines (2 for *UGPase* amiRNA, 3 for *INV* amiRNA and 2 for *UGPase*-TGS) showed improvement in chip colour compared to wild type control directly after 45 days of cold storage at 5 °C; while 12 transgenic lines (3 for *UGPase* amiRNA, 2 for *INV* amiRNA, 1 for *UGPase-INV* combined amiRNAs and 5 for *UGPase*-TGS) showed improvement in chip colour after 21 days of reconditioning.

Conclusion
Discovery and understanding of the mechanism and function of RNA-mediated gene silencing have ushered its practical use in functional genomic studies and crop improvement. Because of robustness in efficacy, PTGS and TGS are now the preferred tool of development of gene knock-down mutants to decipher gene functionality, virus resistant transgenics, and of qualitative crop improvement by silencing undesirable genes. miRNA has provided biologists deep insight into the regulation of biological processes in plants. We have just started

appreciating the idea of manipulating miRNA-mediated yield determining processes for crop improvement and future beacons with prospects.

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Development of Proteomics Technique and its Utilization in Plant Pathology

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Introduction

Plants respond to various environmental conditions like biotic and abiotic stress by modulating their gene expression pattern. Obviously proteins, the final products and functional representatives of the genome, are the workhorses in the biological system. Proteins whose expression is regulated by pathogen attack or treatment with pathogen elicitors are referred to as defense related proteins or pathogenesis related (PR) proteins. Inducible defense-related proteins have been described in many plant species upon infection with oomycetes, fungi, bacteria, or viruses, or insect attack. Several types of proteins are common and have been classified into 17 families of pathogenesis-related proteins (PRs) (Van et al 1999). Others have so far been found to occur more specifically in some plant species. Most PRs and related proteins are induced through the action of the signaling compounds salicylic acid, jasmonic acid, or ethylene, and possess antimicrobial activities in vitro through hydrolytic activities on cell walls, contact toxicity, and perhaps an involvement in defense signaling. Many genes encoding defense related proteins have been introduced into susceptible plants by transgenic approach to improve the disease resistance capacity (Grover et al., 2003).

Transcriptomics and proteomics are the two major classes of functional genomics research. DNA microarray and 2-D protein electrophoresis are the two major techniques used for the high throughput analysis of transcriptomes and proteomes respectively at a give point of time in different tissues or stress conditions. These approaches provide a global snapshot of genes associated with plant defense mechanism. Examination of the transcriptome gives an accurate indication of which genes are active in a particular cell, but gives a less accurate indication of the proteins that are present. Proteins, not the genes themselves, are the key components that actually perform essential functions in the cell. There are several reasons for this lack of equivalence between transcriptome and proteome viz., the protein content of the cell is determined by both synthesis of new proteins and degradation of existing ones, not all mRNAs are actively translated at any particular time and posttranslational modifications such as phosphorylation and glycosylation are vital for cellular functions. So the study of proteins is important to establish the role of a particular protein in the stress response/defense mechanism (Zivy et al., 2000). Proteomics deals with the characterization of the entire protein complement of the genome at a given point of time under the specific physiological condition and identifies differentially expressed proteins. The methodology used to study proteomes is collectively called proteomics. 2DE is the only most efficient technique by which majority of the proteins in a particular sample can be separated and quantified. It involves five major steps viz., (i) Sample preparation (ii) Isoelectric focusing (iii) SDS-PAGE (iv) Visualization/ image analysis and (v) mass spectrometry based characterization of protein spots of interest.

2DE - an overview

As each individual protein in a sample has its own unique aminoacid sequence and number of aminoacids, each protein will have unique pI and molecular weight. Two dimensional electrophoresis (2DE) sorts proteins according to two independent properties in two individual steps: in the first-dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI); in the second-dimension step, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights. Each spot on the resulting two-dimensional gel corresponds to a single protein species in the

sample. Thousands of different proteins in a sample can thus be separated in a single gel, and the properties such as the protein isolectric point (pI), the apparent molecular weight, and the amount of each protein is obtained.

2DE was first developed and introduced by O'Farrell and Klose in 1975. In that time the first dimension IEF separation was carried out in polyacrylamide gels containing carrier ampholytes. Narrow plastic tubes were used to cast these gels, after first dimension step, the focused gels were carefully taken out and placed on the PAGE gel for second dimension. But handling the tube gel is a very frustrating job and needs more skilled workers.

Use of 2DE in biological research was started virtually since its introduction, but its wide scale usage was started during the last decade due to the improvements both in the instrumentation and reagents. The major improvements which helped in wide scale usage of 2DE include (i) introduction of immobilized pH gradients by Bjellqvist et al., in 1982 (ii) Development of immobilized pH gradient strips (IPG strips), and in that tube gels were replaced with gel supported by a plastic backing (Gorg et al. 1988). (iii) Development of new mass spectrometry techniques like MALDI-TOF (iv) Development of sophisticated image analysis softwares for the rapid and accurate identification of differential spots among multiple gels (v) availability of genome and transcriptome sequence information which helps in the interpretation of mass spectrometry results and in the characterization of protein spot.

Basic principle in 2DE

The 2DE electrophoresis is based on the two major techniques viz., Isoelectric focusing (IEF) and SDS-PAGE. IEF is an high voltage electrophoretic method that seperates proteins according to their isoelectric points (pI). The pI is the pH at which the net charge of the protein is zero. The pI of each protein is unique due to the composition of aminoacids. Each protein carry either positive, negative, or zero net charge, depending on the pH of their surroundings. Proteins arepositively charged at pH values below their pI and move towards negative electrode. At pH values above the pI, proteins are negatively charged and tend to move towards the positive electrode. At pH values equal to their pI, proteins posses no net charge and stops migrating. IEF utilizes the above property for the separation of proteins, where protein sample is loaded on to an immobilized pH gradient strip (which is having gradient of pH environment) and high voltage is applied. The result is the high resolution separation of proteins based on their pI. In second step the IEF separated proteins were further subjected to SDS-PAGE electrophoresis to separate them on the basis of size (Mr). For second dimension the electrofocused strip is carefully placed on the top of the SDS-PAGE gel and sealed with agarose solution, after that the proteins were separated as in the regular SDS-PAGE technique. The resulting gel, after staining, will have protein spots instead of bands due to the two dimensional separation. Each spot in the gel is the representative of a single protein entity in the sample. If two samples are ran in two separate gels under same experimental conditions, they can be compared and the differentially expressed spot can be identified.

Critical steps in 2DE

Sample preparation is the most important step in 2DE. As the proteins from a given sample belong to diverse classes and types, a single extraction protocol cannot efficiently extract all the proteins in a sample. So the best sample preparation procedure should have the efficiency to extract as much possible proteins present in the sample. The most commonly used method is the TCA/Acetone precipitation followed by protein solubilization using lysis buffer containing urea. The second commonly used method is the phenol extraction. Both methods have their own advantages and disadvantages, so one have to be very careful while selecting the suitable protocol for sample preparation. Developments in the past decade resulted in the introduction of sub cellular proteomic analysis, in which proteins from a

specific organ/source is extracted using specialized protocols. Membrane proteomics is the best example for such kind of techniques. Apart from extraction, the good sample preparation protocol should be able to remove non protein contaminants such as phenolics, nucleic acid and polysaccharides etc. Usually the best protocol for a given sample has to be empirically determined depending upon the source tissue type, possible contaminants and objective of the study. The next important step is the protein visualization using staining techniques. CBB and silver staining are the widely used visualization techniques in 2DE. SybroRuby also become frequently used now a days due to its high dynamic range and ease of handling. Silver staining is particularly suitable in situations where the amount of sample for analysis is a limiting factor. CBB is less sensitive than silver staining but has several advantages like good dynamic range, low in keratin contamination, Mass spectrometry compatible and only a few steps involved. DIGE, a fluorescent based staining technique can able to separate proteins from two or three samples in a same gel, where the proteins from different samples were labeled with different dyes and the samples were then mixed in equal quantity prior to 2DE. DIGE technique has greatly enhanced the reproducibility of the 2DE technique, which is otherwise has comparatively less reproducibility. And also the image analysis is also very simple in case of DIGE gels as in the microarray data analysis. The third critical step is the selection appropriate IPG strips, due to rapid advances in the field now IPG strips are available in various pH ranges and sizes. Again the selection of a suitable strips has to be empirically determined based on the abundance of proteins in a particular pI range and also based on the objective of the study. If the entire proteins needs to resolved in a single gel, the pH range of 4-7 and the strip length of 18cm or above is good. But usually researches found the use of multiple IPG strips with narrow pH range gives the maximum resolution and reproducibility than the wide range strips.

Mass Spectrometry and proteomics

Mass spectrometry identifies the chemical composition of a compound or sample, in protein identification studies the protein/peptide of interest is first fragmented into charged particle by ionization techniques like electrospray (ESI) or matrix-assisted laser desorption/ionization (MALDI) (Siuzdak, 1996). Upon ionization, molecules either gain or lose their charge and then they are electrostatically propelled inside the detector. The time taken by each molecule to reach the detector is based on the mass to charge ratio (m/z). So, for a given peptide mixer the mass spectrometric analysis results in the identification molecules with different m/z ratios, this is otherwise called peptide mass fingerprint (PMF). By comparing the PMF spectrum of the unknown protein with the PMF of previously known one results in the identification of the unknown protein. Alternatively it is possible to make virtual PMF by using the translated nucleotide sequences and then we can use them to compare with our unknown protein. Now a days, there are so many databases are available for the PMF analysis using nucleotide sequences as query, ExPaxy and SwissProt are the two major database used for this purpose. Developments in the DNA sequencing methodologies enabled the accumulation of huge volume of genomic and transcriptomic sequence in the past two decades, which also helped the identification of unknown proteins easily using mass spectrometric techniques. Introduction tandem mass spectrometry (MS/MS) is also helped in the improvements in the protein identification. In MS/MS, a particular ion is selected with a mass filter and then further fragmented and analyzed. The fragmentation is achieved by the collision of ions with a collision gas such as argon atoms, the resulting sub ion spectrum is then analyzed (Siuzdak, 1996). Mass spectrometry also resulted in the development of advanced/highthoughput proteome analysis technique which can complement the 2DE. MudPIT, iTRAQ, SILAC, ICAT and LOPIT are the recently developed techniques in proteomics. In MudPIT (multidimensional protein identification technology) the protein separation is carriedout in a reversed phase liquid chromatographic instrument that is directly coupled to tandem mass spectrometric instrument (Washburn et al., 2001). MudPIT analysis is easy to perform, less time consuming method when compared to 2DE. But the

interpretation of MudPIT results is the very difficult process which needs much expertise and also the sophisticated softwares. So non gel based techniques like MudPIT cannot replace 2DE, but rather they can compliment 2DE for the improved proteome coverage.

Application of proteomics in plant pathology research

Plant pathogens such as bacteria, fungi and viruses are causing devastating crop losses worldwide. Understanding the mechanisms and network of changes happening during the host pathogen interaction has always been the focus of plant pathologist. Understanding the mechanisms/key components involved in pathogenesis and incompatible interactions will definitely help us to evolve disease resistance varieties and also to device precise/safe and economical management strategies. Even though various tools like classical biochemical, genetic, genetic and microbiological techniques were used to understand the mechanisms, molecular biological tools such as genomics and proteomics are important to provide holistic picture of various changes occur at molecular and cellular level. Proteomic based techniques are currently being used for the analysis of proteome changes in the host plants during interaction with their pathogens. Proteome changes in pathogens are comparatively less studied. The amount of data available from the proteomic studies is less when compared to genomic data as the host pathogen proteomics is still in the blossoming stage. But definitely there are several examples where these techniques are successfully employed to study plant pathogen interaction.

In plant bacteria interaction, analysis of pathogen secretome is very important due to the involvement of secreted proteins in the infection process. For example, analysis of secretome of E.chrysanthemi in the presence of Chrysanthemum leaf extracts revealed the up-regulation of several pectate lyases and polygalacturonase enzymes which are involved in the cell wall degradation (Pour et al.2004). Jones et al. (2004) were identified the differential expression of glutathione S-transferases and peroxiredoxins in Arabidopsis thaliana leaves upon challenge with *Pseudomonas syringae pv.* tomato. Mahmood et al. (2006) applied this approach to identify bacterial-blight defence responsive proteins in rice leaf blades.

Rice blast caused by the fungus *Magnaporthe grisea* is the most severe and also most extensivel studied plant fungi interaction. The genome of the rice blast fungus is completely sequenced (Dean et al. 2005). Now the major task is to decode the genome information with help of functional analysis tools like transcriptomics and proteomics to understand the molecular mechanisms rice-blast interaction. So many studies were conducted with this objective involving proteomic tools. Kim et al. 2004(a) identified four important proteins involved in the appressorium formation during infection of *M.grisea*. On the host side, induction of receptor like protein kinases, glucanase and thaumatin-like protein were revealed by 2DE approach (Kim et al 2004b). Santo et al 2010 identified several important proteins specifically expressed during endophytic interaction of *Gluconacetobacter diazotrophicus* with sugarcane. Lee et al. 2009 employed a gel free, high throughput liquid chromatography tandem mass spectrometry method to study the differences between basal and R-gene mediated defense response among resistant and susceptible bean cultivars during interaction with *Phaseolus vulgaris*. They concluded that R-genes actually are components of basal defense mechanism and reinforce the basal defense components weakened by the fungus. Chivasa et al., 2006 used 2DE-DIGE method to identify fungal elicitor induced proteins in Arabidopsis suspension cultures.

Proteomic tools are also used to reveal the network of events happening during plant viral interactions. For example, Debout et. al. 2004 identified several new proteins which were differentially induced between resistant and susceptible cultivars during infection with Rice yellow mottle virus. Cooper et al. (2003) were able to identify a new/previously uncharacterized virus by using 2DE technique and concluded that the technique can be utilized in the discovery of new viruses.

Proteomics work at SBI, Coimbatore

Proteomic work in sugarcane is very limited and poorly utilized. Sugarcane tissues are known for their rigidity, fibrous nature, extreme levels of oxidative enzymes, phenolic compounds, carbohydrates especially sucrose, and other interfering metabolites especially in the stalk tissues. Very few reports are available on protein analyses in sugarcane. Among the research articles published on sugarcane dealt only the proteins extracted from the leaf tissues. But the pathogen of our interest, *Colletotrichum falcatum* is a stalk pathogen and necessitates the need to device a suitable method for protein extraction from the stalk tissues. We compared five different protein extraction methods viz., Direct Lysis (LB), TCA precipitation with Lysis (TCA-LB), Lysis buffer with Thiourea (TCA-LBT), Lysis buffer with Thiourea and tris (TCA-LBTT) and phenol extraction for their suitability in 2-DE. The TCA/acetone followed by solubilization in LB containing thiourea (LBT) extraction method and the phenol method were found to be suitable methods for stalk proteome analysis. Even though the LBT method gave good quality proteins, number of spots was found to be less when compared to the phenol extraction method. Despite the fact that phenol method produced higher number of spots and good quality of proteins, it resulted in the shift in protein pI values. To overcome this problem, a 2-D cleanup kit was used. In phenol method, the interfering substances such as salts and carbohydrates were removed from the sample using the cleanup kit. From the three 2-D gel profiles obtained using LB, LBT, and phenol protocols compared above, a total of 30 common protein spots (from 136 common spots) were randomly selected for MS/MS analysis. MS/MS analysis revealed that, among the 30 identified common spots 23% were found to be involved in defense mechanisms and 23.33% were found to have key roles in sugar phosphate metabolism. Apart from these majority groups, proteins involved in sugar metabolism (20%), nucleic acid metabolism (13.33%), protein metabolism and cell growth and development (10%), structure and defense (6.67%), secondary metabolism (6.67%), were also identified. About 16.67% proteins were unknown or uncharacterized. Using the above results, a representative sugarcane stalk proteome reference 2-D gel map was created (Ramesh et al., 2010). This is a very first basic study on sugarcane stalk proteome analysis and will help promote the unexplored areas of sugarcane proteome research.

By adapting the above standardized protocols, important proteins which are differentially regulated during sugarcane red rot interaction were identified. Briefly, protein was extracted from healthy, mock inoculated and red rot spore inoculated stalk tissues of two sugarcane varities viz., CoC 671(susceptible to red rot) and 93009 (resistant to red rot) and subjected to 2DE. The number of protein spots was found to be higher (335 ± 7) in the resistant cultivar after 12 h of pathogen challenge. The inoculated susceptible cultivar had the lowest number of protein spots (280 ± 3) (Fig-1& 2). More than 250 protein spots that were detected in stalk tissues by proteomic analysis showed reproducible abundance within the replications. Approximately 50 protein spots were additionally induced in the resistant cultivar upon pathogen inoculation, whereas ~ 24 proteins have got down-regulated in the susceptible cultivar. The results showed the differential regulation of ~ 150 protein spots, of which 125 no of spots were picked out and characterized by MALDI-TOF analysis. Our studies on the proteome-level changes that occur during *C. falcatum* – sugarcane interaction using 2-DE, are the first attempt to standardize proteome analysis and to identify specific proteins involved in red rot resistance in sugarcane (Viswanathan et al., 2010). Similarly studies were conducted to identify SAR induced proteins involved in resistance to *C. falcatum* in sugarcane. Synthetic elicitor BTH treated and water- treated cane samples were subjected to 2-DE. The differentially expressed proteins analyzed by 2-DE approach indicated their possible role in defense against red rot in sugarcane (Nagarathinam, 2010). Overall, our current studies revealed the differential regulation of many important genes involved in the secondary metabolic pathways and phytoalexin synthesis in sugarcane. Some of the identified proteins include putative callose synthase, R2R3-MYB transcription factor MYB6, p-coumarate 3-hydroxylase, PrLTP1 and PISTILLATA-like protein. Further validation of differential expression of the identified proteins by RT-PCR is in progress.

Conclusion

Even though there are huge developments in the field of proteomics, the proteomics application has not been popular as like genomics work. The pathogen proteomics is progressing faster than that of the host proteomics due to the ease of handling and also due to the availability of full genome sequences for most of the pathogens. Of course, so far genomes of more than 25 plant pathogens were completely sequenced. The availability of genome sequences are useful resources for interpreting mass spectrometry results. Studying the pathogen behavior seems to be simpler and a shortcut method to understand the disease mechanism. So, for the beginners, it would be more appropriate to study the pathogen proteome at different conditions rather than the host system. The advancements in the technology like the development of MudPIT will definitely accelerate the proteomic analysis of both pathogen and host systems. In future, a proteomic analysis system comprising both 2DE and gel free/high throughput techniques will become the routine tools for plant pathologist.

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This is one of the routinely used techniques in molecular biology. This is used to separate DNA fragments and to assess the quality and quantity of DNA.

Principle
The gel is made from agarose, a highly purified form of the polysaccharide that is used to make agar plates on which bacteria is grown. The gel is immersed in buffer and the DNA fragments are loaded onto a well at one end of the gel and made to move through the gel by the application of electric current. DNA is negatively charged and so will move towards the positive anode. However, the polysaccharide mix of the gel retards the DNA by a process of sieving, so that small fragments move through faster and these fragments separate according to size.

The DNA is visualised by adding ethidium bromide (EtBr), a fluorescent molecule which intercalate with the DNA bases, extending the length of linear and nicked circular DNA molecules and making them more rigid. When EtBr is added, UV radiation at 254 nm is absorbed by the DNA and transmitted to the bound dye. The energy is re-emitted at 590 nm in the red-orange region of the spectrum. Ethidium bromide is a powerful mutagen and hence the gel should be handled carefully with the gloves. The DNA bands can be visualised under UV and gel documentation appliances can record the data.

Characteristic features of gel electrophoresis are:

1. The molecular weight of the DNA: The migration rate is inversely proportional to the molecular weight
2. Agarose concentration: The migration rate is inversely proportional to the agarose concentration
3. Conformation of the DNA: Linear form travels slowest and the supercoiled form travels fastest
4. Applied voltage: Typical value - 5 volts per cm. The heat generated during electrophoresis is dissipated by the buffer.
5. DNA being polyanionic at neutral pH, it migrates towards the anode.
6. The loading dye for DNA contains glycerol, which gives density to help the sample sink to the bottom of the well and marker dyes Xylene Cyanol and bromophenol blue. Bromophenol blue moves on par with 300-400 bp DNA and Xylene cyanol with 2-3 kb DNA.
7. The DNA is visualised by adding EtBr a fluorescent molecule that intercalates with the DNA bases. To 0.8% agarose gel add EtBr to give 0.5 pg/ml concentration. UV radiation at 254 nm is absorbed by the DNA and transmitted to the bound dye. The energy is re-emitted at 590 nm in the red-orange region of the spectrum.
8. EtBr is a powerful mutagen. The dye is usually incorporated into the gel or conversely the gel is stained after running by soaking in a solution of Et. Br.
9. The usual sensitivity of detection is 0.1 pg of DNA
10. The gel will be run along with a molecular weight marker, a wide range of which is commercially available.

Protocol
1. Prepare 1% agarose gel in Tris-acetate EDTA buffer (IX TAE) containing EtBr
2. To 1 gm of agarose, add 100 ml of IX TAE. Heat until dissolved. Cool the gel to 50°C and add EtBr (0.5 pg/ml) before pouring into the gel apparatus.
3. Wash the gel casting tray and comb with water to remove dirt.
4. Place the apparatus on a level surface and check with the spirit level and adjust the level.

5. Choose appropriate comb (commonly 12 slots) and fix into position.
6. Pour the gel onto the apparatus and allow it to cool and set.
7. After the gel has set firmly, pour little amount of buffer and remove the comb gently. Take care not to drag the comb and break the gel.
8. Immerse the gel slowly into the gel tank. Add sufficient amount of IX TAE buffer. Connect the electrode and check the current.
9. *Note*: Always check the electrical connections before loading the sample.
10. Load the samples into wells carefully.
11. Always load an aliquot of standard molecular weight marker along with the samples. It will help in assessing the size of the DNA fragment by comparing with the electrophoretic mobility.

Anticipated Results

Depending upon its confirmation and size, the DNA fragments will move as discrete bands. The EtBr intercalated with the DNA fragments will make it emit pink fluorescence under UV lamps, which can be recorded in any gel documentation system.

Quantitation of DNA

Reliable measurement of DNA concentration is important for many applications in molecular biology including complete digestion of DNA by restriction enzymes and amplification of target DNA by polymerase chain reaction. DNA quantification is generally carried out by spectrophotometric measurements or by agarose gel analysis.

Principle

The purines and pyrimidines in nucleic acid show absorption maxima around 260 nm (dATP 259, dCTP 272; dGDP 253, dTTP 247) of the DNA samples is pure without any contamination of protein or organic solvents vs. absorption at 260 nm can be taken to correctly assess the quantity. If the sample amount is less it can be visually estimated by gel electrophoresis.

Protocol

(a) Spectrophotometric measurements

1. Take 1 ml TE buffer in a cuvette and calibrate the spectrophotometer at 260 nm as well as 280 nm wavelength.
2. Add 2 to 5 pi of DNA, mix properly and record the optical density (OD) at both 260 and 280 nm.
3. Estimate the DNA concentration employing the following formula
4. Amount of DNA ($\mu g/\mu l$) = $\dfrac{(OD)_{260} \times 50 \times \text{dilution factor}}{1000}$

5. Judge the quality of DNA from the ration of the OD values recorded at 260 and 280nm. The A_{260}/A_{280} ration around 1.9 (1.85 – 1.95) indicates best quality of DNA.

Given below are the methods for preparing buffers and reagents, which are generally used in a molecular biology laboratory. The solutions, which are specific for any experiment, are given under the respective headings.

30% Acrylamide

Acrylamide	30.0 g
N-N'methylene bisacrylamide	0.8 g

Dissolve in distilled water and make up the volume to 100 ml. Store in a dark bottle at - 4°C for up to 2 weeks. Acrylamide is neurotoxic. Wear gloves while handling.

10% Ammonium persulphate (APS): Dissolve 1 g of APS in sterile distilled water and make up to 10 ml. Store at 4°C. It is preferable to prepare freshly.

Antibiotics: Dissolve 5 mg of ampicillin/kanamycin in 10 ml of sterile double distilled water. Filters sterilize and dispense into 200 μl aliquots and store at -20°C.

Antibody diluent/buffer (for ELISA)

Add 2g polyvinyl pyrrolidone (PVP) and 0.2g ovalbumin to 100 ml of PBS-T

Blocking solution (for ELISA)

Add 5.0 g Bovine Serum Albumin (BSA)/Spray dried milk (SDM) to 1 litre PBS-T.

Stop solution (for ELISA)

NaOH	12.0 g
Distilled H_2O to make	1 litre

All buffers contain 0.02% sodium azide as a preservative.

100 mM CaCl$_2$

Add 1.47 g of CaCl$_2$. 2H$_2$O in H_2O. Make upto 100 ml. Sterilise by autoclaving.

Catalase (CAT)

Staining:

0.01%H_2O_2	75 ml

Stain solution:

Potassium ferricyanide	0.375g
Ferric chloride	0.375g
Volume is made up to	75 ml.

7% acetic acid

Chloroform:Isoamyl alcohol (24:1), 100ml

Mix 96ml of chloroform and 4ml of isoamyl alcohol. Mix well and store in reagent bottles at room temperature. Chloroform evaporates, so the cap should be tightly closed.

Coating buffer (Carbonate buffer, pH 9.6)

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g

Distilled H_2O to make 1litre

CPG (Cassamino Peptone Glucose) medium:

Tryptone	10g
Glucose	5g
Cassamino acid	1g
Yeast extract	1g
Agar	18g

Tetrazolium chloride (10mg/ml) 500ul/100ml (pH 7.2)

Use CPG for mass culturing *Ralstonia solanacearum*

DNA extraction buffer for soil DNA

Tris-Cl (pH 8.0)	100mM
EDTA (pH 8.0)	100mM
Na2H Po4 (pH 8.0)	100mM
NaCl	1.5M
CTAB	1%
Proteinase K	20μg
Lysozyme	100μg

DNA extraction buffer for plants

Tris Cl pH 8	100 mM
EDTA pH 8	20 mM
NaCl	1.4 M
2- mercaptoethanol	0.1%
CTAB	2%

DNA Molecular Weight Marker

Commercial molecular weight markers are normally prepared from digestion of different plasmids to produce discrete fragments.

DNA (ladder)	100 µl
50 x TAE	5 µl
6 x Gel loading dye	42 µl
SDW	103 µl
Total volume	250 µl

The marker is ready to be loaded.

Denaturing PAGE

Urea (AG)	453 g
10X TBE	50 ml
40%(19:1 ratio)	
Acrylamide : bisacrylamide	125 ml
Distilled water (make up to final vol of 1 litre)	

Destaining solution

Methanol	50.0 ml
Glacial acetic acid	75 ml
Distilled water	1000 ml

Diaphorase (DIA)

Stain solution :	75ml
NADH	37.5mg
MTT	9mg
DCPIP	1.5mg
Tris-Cl, pH 8.0	7.5ml (Volume is made up with distilled water).

0.5 M EDTA (pH 8.0) : Add 186.1 g of disodium ethylene diamine tetraacetate. $2H_2O$ to 800 ml of H_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH. Make upto 1 litre. Dispense into aliquots and sterilize by autoclaving.

Ethidium Bromide (10 mg/ml) : Add 1 g of ethidium bromide to 100 ml of H_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminium foil or transfer to a dark bottle and store at 4°C. **Caution**: Ethidium bromide is a mutagen and toxic.   Wear gloves when working with ethidium bromide solutions and a mask when weighing it out.

70% ethanol, 100ml

Mix 70ml of ethanol and 30ml of distilled water. Dispense to reagent bottles and store at 4^0C or -20^0C.

Equilibriation of Phenol: Melt 500 g phenol in a water bath, kept at 65°C. Add 100 ml of 100 mM Tris Cl (pH 8.0) and shake to emulsify. Let phases separate. Repeat this step until the aqueous layer shows a pH between 7.0 to 7.5. Store the phenol under 50 ml of 10 mM Tris HCl pH 7.6 in an amber coloured bottle.

Enzyme conjugate diluent/buffer (PBS-T polyvinyl-pyrrolidone ovalbumin, PBS TPO) (for ELISA): Add 20.0 g polyvinylpyrrotidone (PVP, MW 44,000) and 2.0 g egg ovalbumin to 1 litre PBS-T.

<u>Fixative solution</u>

Methanol	400 ml
Glacial acid	70 ml
Distilled water	1000 ml

GYP Medium (1L) for *Phytophthora capsici*

L.Asparagine	20gm
$FeSO_4 7H_2o$	20mg
$CaCl2.2H_2o$	10mg
$MgSO4.7H_2O$	0.1gm
KH_2PO_4	0.47gm
$K_2H_2PO_4$	0.26gm
Thiamine hydrochloride	10mg
$ZnSO_4.7H_20$	1 µg/ml
$CuSO_4.5H_2O$	0.02µ/ ml
$Mncl_2$	2mg
pH adjusted to 6	

Isocitrate dehydrogenase
Stain solution : 75ml

Isocitric acid	41.6mg
$MgCl_2$	166.6mg
NADP	33.3mg
NBT	8.3mg
PMS	1.7mg

1.0M Tris-Cl, pH8.0   7.5ml

Volume was made up with distilled water

Malate dehydrogenase (MDH)

Stain solution : 75ml

2.0 M Malic acid   2011.35mg (2.0M) dissolved in a small qty. of water.

      (pH adjusted to pH 8.0 with NaOH)

NAD   33.3mg

NBT   16.65mg

PMS   3.33mg

1.0M Tris-Cl, pH8.0   7.5ml

Volume was made up with distilled water

PVPH incorporated Carrot Agar (1L)

Carrot   200gm

Agar   20gm

Volume made upto 1000ml using sterile distilled water

For every 100 ml of carrot agar 10ml of PVPH was added.

LA medium (Luria agar medium): 950 ml of deionized water, add 10 g of bactotryptone, 5 g of yeast extract and 10 g of NaCl and 15 g of agar. Boil to dissolve content, adjust pH to 6.8 with 5 N NaOH and make up volume up to 1 litre with deionized water. Sterilize by autoclaving.

Luria-Bertani broth: It is same as above but prepared without agar.

Loading dye (6X)

Tris-HCl, pH 8.0   50 mM

EDTA   5 mM

Glycerol   25%

Bromophenol blue   0.2%

Xylene Cyanol FF0.   2%

This loading dye is used for Non-denaturing ds DNA

<u>6X Loading dye</u>
1% Bromophenol blue   200 µl

Glycerol   200 µl

10% SDS   60 µl

0.5 M EDTA	50 µl
10 X TAE	60 µl
Water	30 µl

100 mM MgCl$_2$: Add 2.03 g of MgCl$_2$.6H$_2$O in H$_2$O and make upto 100 ml. Sterilise by autoclaving.

5M NaCl : Dissolve 292.2 g of NaCl in H$_2$O and make upto 1 litre. Dispense into aliquots. Sterlize by autoclaving.

Nutrient agar (NA)

Beef extract	5 g
Peptone	10 g
NaCl	5 g
Agar	20 g
Distilled water	1000 ml (pH 7.0)

Nutrient broth (NB) : Same as NA, but without agar.

Plasmid isolation buffers (Modified alkaline lysis method)

Solution I	:	25 mM Tris HCl (pH 8.0)
(resuspension buffer)		50 mM Glucose
		10 mM EDTA
Solution II	:	0.2 N NaOH
(lysis buffer)		1 % SDS
Solution III	:	3 M Sodium acetate
(neutralization buffer)		(pH 4.8)

Phenol: Chloroform(1:1)

Mix equal amounts of phenol and chloroform. Equilibrate the mixture by extracting several times with 0.1M Tris.Cl (pH 7.6). Store the equilibrated mixture under an equal volume of 0.01M Tris.Cl (pH 7.6) at 4^{0}C in dark glass bottle.

Phosphate buffer-saline, PBS, pH 7.4

NaCl	8.0 g
Na$_2$HPO$_4$.2H$_2$O	1.44 g or Na$_2$ HPO$_4$.12H$_2$O 2.90 g or Na$_2$HPO$_4$ 1.50 g
KH$_2$PO$_4$	0.20 g
KCl	0.20 g

Distilled water to make 1 litre

PVS 2 solution pH 5.8

Glycerol	30% v/v
Ethylene glycol	15% v/v
DMSO	15% v/v
Sucrose	0.4 M

Resolving gel solution

Acrylamide stock solution	19.8 ml
Tris-SDS stock solution (pH 8.8)	30 ml
Distilled water	to 60 ml
TEMED	15 µl
10 % APS	1 ml

RNAse (10 mg/ml)

Dissolve RNAse A at a concentration of 10 mg/ml in 0.01 M sodium acetate(pH 5.2). Heat to°C for 15 minutes. Allow to cool slowly to room temperature. Adjust the pH by adding 0.1 volumes of 1 M tris-Cl(pH 7.4). Dispence into aliquots and store at -20°C.

10% Sodium dodecyl Sulfate (SDS) : Dissolve 100 g of SDS in K_2O and make upto 1 litre. Dispense into aliquots.

3M Sodium acetate (pH 4.8 and 5.2) : Dissolve 408.1 g of sodium acetate $3H_2O$ in 800 ml of distilled water. Adjust the pH to 4.8 to 5.2 with glacial acetic acid. Make upto 1 litre. Dispense into aliquots. Sterilize by autoclaving.

Staining solution

Coomassie Brilliant Blue R 250	1.15 g
(dissolved in 227 ml methanoi)	
Glacial acetic acid	46 ml
Distilled water	500 ml

Sample buffer

Tris-SDS stock pH 6.8	25 ml
SDS	2.0 g
Glycerol	10 ml
2-mercaptoethanol	5.0 ml
1% bromophenol blue	0.1 ml
Distilled water	100 ml

Stacking gel solution (for two 1.5 mm gel, 3%)

Acrylamide stock solution	2 ml
Tris-SDS stock sol (pH 6.8)	10.0 ml
Distilled Water	60 ml
TEMED	70 µl
10 % APS	1 ml

1M Sucrose

Dissolve 34.23g of sucrose in 80ml of water. Make up the volume to 100ml. Filter sterilize.

Superoxide dismutase (SOD)

Stain solution : 75ml

Na_2EDTA	5.625mg
Riboflavin	3.0mg
NBT	7.5mg
1M Tris-Cl, pH8.0	3.75ml (0.5M final concentration)

Volume is made up with distilled water.

1M Tris-HCl : Dissolve 121.1 g of Tris hydroxylmethane aminomethane in 800 ml distilled H_2O. Adjust the pH to 8.0 with concentrated hydrochloric acid. Make upto 1 litre. Dispense into aliquots.   Sterilise by autoclaving.

500ml, Tris-Cl, 1M pH-8.0

Dissolve 60.55gm of base in 400ml H_2O. Adjust pH to 8.0 using conc. HCl (approx.21ml). Allow to cool the solution to room temperature and do final adjustments with pH. Make up to 500ml. Filter and sterilize by autoclaving. Store at 4^0C. pH of this solution is temperature dependent. Tris will completely dissolve only when pH becomes 8.0. Initial pH will be around 11

Tris-buffer saline, TBS, pH 7.5

0.02 M Tris	4.84 g
0.5 M NaCl	58.48 g

Adjust the pH to 7.5 with 1 N HCl and make upto 2 litre. This is used as Wash solution.

Tris-Glycine-SDS electrode buffer, pH 8.3

Tris	15.15g
Glycine	72.0 g
SDS	5.0 g

Dissolve in water and make up the volume to 5000 ml.

Tris-SDS stock solution pH 6.8

Tris   39.4 g

SDS   2 g

Dissolve in 900 ml distilled water. Adjust pH to 6.8 with HCL and make up the volume to 1000 ml.

Tris-SDS stock solution, pH 8.8

Tris   118.2 g

SDS   2 g

Dissolve in 900 ml distilled water. Adjust pH to 8.8 with HCI and make up the volume to 1litre.

10X TBE (500 ml)

Tris   54 g

Boric acid   27.5 g

EDTA (sodium salt)   4.65 g

TE saturated phenol : chloroform : isoamyl alcohol : To the equilibriated phenol add equal amount of chloroform : isoamyl alcohol (24:1). Store at 4°C in an amber coloured bottle under a layer of 10 mM Tris-CI (pH 7.6).

50 X TAE : Dissolve 242 g of Tris in H_2O. Add 57.1 ml of glacial acetic acid and add 100 ml of 0.5 M EDTA (pH 8.0). Make upto 1 litre. Dispense into aliquots. Sterilise by autoclaving.

Wash buffer (PBS-Tween, PBS-T) (For ELISA) : Add 0.5 ml Tween - 20 to 1 litre PBS.

Common Conversions of Nucleic Acids and Proteins

Weight conversion

1 µg	=	10^{-6} g
1 ng	=	10^{-9} g
1 pg	=	10^{-12} g
1 fg	=	10^{-15} g

Spectrophotometric conversion

1 A_{260} unit of double-stranded DNA = 50 µg/ml

1 A_{260} unit of single-stranded DNA = 33 µg/ml

1 A_{260} unit of single-stranded RNA = 40 µg/ml

DNA molar conversions

1 µg of 1,000 bp DNA – 1.52 pmole (3.03 pmoles of ends)

1 pmole of 1000 bp DNA = 0.66 µg

Protein molar conversion

100 pmoles of 100,000 dalton protein = 10 µg

100 pmoles of 50,000 dalton protein = 5 µg

100 pmoles of 10,000 dalton protein = 1 µg

Protein/DNA conversion

1 kb of DNA = 330 amino acids of coding capacity – 3.7 x 10^4 dalton protein

10,000 dalton protein = 270 bp DNA

50,000 dalton protein = 1.35 kb DNA

100,000 dalton protein = 2.7 kb DNA

Average weight of DNA base pair (sodium salt) = 650 daltons

MW of a double-stranded DNA molecule = (# of base pair)' (650 daltons/base pair)

Moles of ends of a double-stranded DNA molecule = 2' (grams of DNA)/(MW in daltons)

Moles of ends generated by restriction endonuclease cleavage:

1. Circular DNA molecule: 2 x (moles of DNA) x (number of sites)
2. Linear DNA molecule: 2 x (moles of DNA) x (number of sites) + 2 x (moles of DNA)

1.0 kb DNA = coding capacity for 33 amino acids = 37,000 dalton protein

10,000 dalton protein   = 270 bp DNA

50,000 dalton proteins   = 1.35 kb DNA

Linear DNA	µg/ml	Moles/ml	Molecules/ml	Conc.	Conc.5' ends
1.0 A_{260} DNA =	50µg/ml	1.6×10^{-12}	9.8×10^{11}	1.6nM	3.2nM
1.0 A_{260} pBR 322 DNA =	50µg/ml	1.8×10^{-11}	1.1×10^{13}	18.0nM	36.0nM
1.0 A_{260} linker (8 MER) =	50µg/ml	9.8×10^{-9}	5.9×10^{15}	10.0µM	20.0µM

Formulae

Picomole ends per microgram of double-stranded linear DNA

$(2 \times 10^{6}) /(660 \times$ Number of Bases) = pmole ends/µg

Exact molecular weight of an oligonucleotide
[(A x 312.2) + (G x 328.2) + (C x 288.2) + (T x 303.2) – 61.0]

=   MW (g/mol) of specific oligonucleotide

Codon Usage Table

	U	C	A	G	
	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
U					
	UUA Leu	UCA Ser	UAA Stop (och)	UGA Stop (opal)	A
	UUG Leu	UCG Ser	UAG (stop (amb)	UGA Trp	G
	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
C					
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
	AUU lle	ACU Thr	AAU Asn	AGU Ser	U
	AUC lle	ACC Thr	AAC Asn	AGC Ser	C
A					
	AUA lle	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met	ACG Thr	AAG Lys	AGG Arg	G
	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
G					
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val (Met)	GCG Ala	GAG Glu	GGG Gly	G

Bases are given as ribonucleotides. GUG usually codes for valine, but it can code for methionine to initiate an mRNA chain. Stop (och) refers to the ochre termination triplet and Stop (amb) refers to the amber.

Amino Acid abbreviations and molecular weights   *Annexure VI*

Amino acid	Three-letter Abbrev.	One-letter Symbol	MW
Alanine	Ala	A	80
Arginine	Arg	R	174
Asparagine	Asn	N	132
Aspartic acid	Asp	D	133
Asparagine/aspartic acid	Asx	B	-
Cysteine	Cys	C	121
Glutamine	Gln	Q	146
Glutamic Acid	Glu	E	147
Glutamine/glutamic acid	Glx	Z	-
Glycine	Gly	G	75
Histidine	His	H	155
Isoleuicne	lle	I	131
Leucine	Leu	L	131
Lysine	Lys	K	146
Methionine	Met	M	149
Phenylalanine	Phe	F	165
Proline	Pro	P	115
Serine	Ser	S	105
Threonine	Thr	T	119
Tryptophan	Trp	W	204
Tyrosine	Tyr	Y	181
Valine	Val	V	117

Archives and Indices

1. Bio Catalog (http://www.ebi.ac.uk/biocat/biocat.html) links to biotechnology-related non-commercial software available via the Internet
2. Indiana University Repository for Biological Software- IUBIO ARCHIVE FOR BIOLOGY (ftp://iubio.bio.indiana.edu)- Indiana University (USA)

DNA Sequence Analysis Software

1. AAT- Analysis and Annotation Tool (http://genome.cs.mtu.edu/aat/aat.html)- Identify genes by comparing cDNA and protein sequence databases
2. AcaClone pDRAW32 (http://www.acaclone.com) - DNA cloning, analysis and visualisation software. Freeware
3. ACD/Protein Manager (http://www.acdlabs.com/products/peptide/prot_mgr.html)- Sequence Analysis tools
4. BioinformatiX (http://www.xpogen.com)- Analysis of microarray data
5. Biopendium (http://www.inpharmatica.co.uk)- Sequence, structure and function relationships for all gene products in the public domain
6. BioTools (http://www.biotools.com)- DNA, protein sequence analysis, and chromatogram analysis
7. BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) - Pair wise sequence alignment
8. CLUSTALW (www.ebi.ac.uk/clustalw/) - Multiple sequence alignment
9. CURVES (http://www.ibpc.fr/UPR9080/Curindex.html)- Nucleic acid helical analysis program
10. DNASIS (http://www.oligo.net/dnasis.htm)- MacOS and Windows-based program for gene analysis
11. Double Digester: (ftp.cs.yale.edu/pub/double_digester)- Yale University (USA)
12. Gene Modeller (Gm) (ftp.tigr.org/pub)
13. FASTA (http://www.ebi.ac.uk/fasta33/)- Pair wise sequence alignment
14. Gene Construction Kit(tm) 2 (http://www.sciquest.com)- A DNA manipulation, cloning design, and illustration tool
15. Gene Inspector(tm) 1.5 (http://www.sciquest.com.)- Sequence analysis package
16. GeneJockey (http://www.biosoft.com/biosoft)- Editing, manipulation, and analysis of nucleic acid and protein sequences
17. GENSCAN (http://genes.mit.edu/GENSCAN.html)- Predicts complete gene structures in genomic sequences
18. GLIMMER (www.tigr.org/software/glimmer/)- uses interpolated Markov models to find genes in microbial DNA
19. Grail(tm) Toolkit. (http://www.apocom.com) - homology-based gene assembly
20. ISYS(TM) (http://www.ncgr.org/isys)- capable of integrating data sources and analysis tools from an investigator's own laboratory
21. Lasergene (http://www.dnastar.com)- sequencing, primer design, sequence alignment, databases and database searching, protein analysis, and restriction map analysis
22. MZEF (http://sciclio.cshl.org/genefinder/)- predicts internal protein coding exons in genomic DNA sequences
23. NIH-Repositories of Sequence Analysis Software (http://molbio.info.nih.gov/molbio/software.htm) - NIH (USA)
24. Onto-Express (www.openchannelfoundation.org)- automates the process of annotating a set of expressed genes with genomic functions
25. OMIGA (http://www.accelrys.com/)- multiple sequence alignments and contains over 50 DNA and protein analysis functions
26. pDRAW32 (www.crosswinds.net/_acaclone)- DNA analysis software by AcaClone software

27. <u>Plasmid Processor</u> (<u>www.hytti.uku.fi/plasmid.html</u>)- You can download full executable version of Plasmid Processor 1.02 for Windows (tm) at University of Kuopio (Finland)
28. Plasmid (http://www.redasoft.com) - vector map drawing and sequence analysis
29. SeqVerter (www.genestudio.com) - nucleotide sequence converter/viewer
30. SIGNALSCAN (http://bimas.dcrt.nih.gov/molbio/signal/)- finds homologies in published signal sequences
31. SRS (http://www.lionbioscience.com/)- database integration and biological information search system
32. TESS (http://www.cbil.upenn.edu/tess/)- locating and displaying transcription factor binding sites in DNA sequences
33. The Rosetta Resolver System (www.rii.com)- high-capacity data storage, retrieval and analysis of gene expression data
34. The Wisconsin Package™ (http://www.accelrys.com)- Sequence analysis contains over 100 interrelated software programs.
35. TurboBLAST (<u>http://www.turbogenomics.com/</u>)- Implementation of BLAST, prvides critical insight into the structure and function of genes and proteins
36. Visual Cloning 2000 (<u>http://www.redasoft.com</u>)- vector map drawing and sequence analysis program

Molecular Modeling Software
1. Antheprot (http://antheprot-pbil.ibcp.fr/Documentation_antheprot.html) - Protein sequence alignment, secondary structure predictions, sites & function detection, physico-chemical profiles, homology search and 3D display of protein structures freely available.
2. Chime (http://www.mdlchime.com/chime/) - Interactively displays 2D and 3D molecules directly in Web pages.
3. Cn3D viewer (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml) - Visualization tool for bio molecular structures in MMDB format, sequences, and sequence alignments
4. Modeler (http://guitar.rockefeller.edu/modeller/modeller.html) - Homology / comparative modeling of protein three-dimensional structures
5. NAB (http://www.scripps.edu/case) - construct models of helical and non-helical nucleic acids
6. Peptool (http://www.biotools.com/) - alignment, homology building, structure prediction
7. PROCHECK (http://www.biochem.ucl.ac.uk/~roman/procheck/procheck.html) - geometric analysis of proteins and DNA including phi-psi angles, hbonding, secondary structure evaluation
8. Rasmol (http://www.umass.edu/microbio/rasmol/) - RasMol is a program for molecular graphics visualization tool for showing the structure of DNA, proteins and smaller molecules
9. Swiss PDB Viewer (http://us.expasy.org/spdbv/) - It is tightly linked to Swiss model, an automated homology modeling server
10. Web lab viewer (http://molsim.vei.co.uk/weblab/) - Create and visualize 3D molecular structures
11. WHAT IF (http://www.cmbi.kun.nl/gv/servers/WIWWWI/) - protein modeling, protein structure analysis, database handler, molecular graphics package
12. Display and analysis of molecular model data (MSC)

Primer Designing Softwares
1. Amplify (<u>http://engels.genetics.wisc.edu/amplify</u>) - Software for PCR - Ver.2.52b
2. Array Designer 2 (http://www.premierbiosoft.com/) - Design hundreds of primers for DNA or oligonucleotide microarrays
3. CODEHOP (http://blocks.fhcrc.org/blocks/codehop.html) - PCR primers designed from protein multiple sequence alignments
4. Fast PCR (http://www.biocenter.helsinki.fi/bi/bare-1_html/oligos.htm) - PCR primer design, DNA and protein tools program

5. GenomePRIDE (http://pride.molgen.mpg.de/genomepride.html) - Primer design program that designs PCR primers or long oligos on an annotated sequence
6. MethPrimer (http://itsa.ucsf.edu/~urolab/methprimer/) - A program for designing bisulfite-conversion-based Methylation PCR Primer.
7. NetPrimer (http://www.PremierBiosoft.com) - Most comprehensive free primer analysis program on the web
8. PCR Rare (http://bioinformatics.weizmann.ac.it/software/PC-Rare) - PC-Rare is a very powerful (but user friendly) software that allows the choice of specific PCR primers.
9. Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) - Pick primers from a DNA sequence
10. Primerfinder (http://eatworms.swmed.edu/~tim/primerfinder/)-Tool to design oligonucleotides suitable for PCR within any sequence
11. Primer Premier 5 (http://www.PremierBiosoft.com/) - Primer design program
12. X-Primer (http://alces.umn.edu/pv/pub/ComBin) - for designing PCR primers - the Virtual Genome Center (USA)

RNA Analysis
1. CARD (rrna.uia.ac.be/card.html) - A computer program for drawing RNA secondary structure models - The rRNA WWW Server - University of Antwerp (Belgium)
2. The RNAdraw Home Page (http://www.yk.rim.or.jp/~aisoai/index.html) - by Ole Matzura
3. RNA Secondary Structure Prediction Software (*ftp.nrcbsa.bio.nrc.ca/pub/*) - NRC (Canada)
4. STRUCTURELAB (http://www-lmmb.ncifcrf.gov/%7Ebshapiro/RNAstructure.html) - Computational Approaches to RNA Structure Analysis
5. Vienna RNA Package (http://www.tbi.univie.ac.at/%7Eivo/RNA/) - The Vienna RNA Package consists of a library and some standalone programs for the prediction and comparison of RNA secondary structures *by* Ivo Hofacker - Wine (Austria)

Systematics and Taxonomy
1. An Alphabetic List of Genetic Analysis Software- This list mainly contains computer software on genetic linkage analysis, marker mapping, and pedigree drawing.
2. DELTA: *ftp* - DEscription Language for TAxonomy (Australia)
3. FastDNAml (http://bioweb.pasteur.fr/seqanal/interfaces/fastdnaml.html) - Construction of phylogenetic trees of DNA sequences using maximum likelihood
4. GeneStudio Pro (http://www.genestudio.com) - Sequence alignments, Phylogenetic analysis and display of trees and Full integration of Internet resources such as Entrez (GenBank), PubMed, and BLAST
5. PHYLIP phylogeny package (http://evolution.genetics.washington.edu/phylip.html) - It is a *free* package of programs for inferring phylogenies
6. Phylodendron (http://iubio.bio.indiana.edu/soft/molbio/java/apps/trees/) - An application for drawing phylogenetic trees, used in evolutionary biology. Options allow you to save in various formats, print, edit, modify, and adorn the tree.
7. TreeGen (http://cbrg.inf.ethz.ch/Server/subsection3_1_6.html) - Compute phylogenetic trees and 2D optimal placements based on distance matrices and optional variances
8. TreeTop (http://www.genebee.msu.su/services/phtree_reduced.html) - Phylogenetic Tree Prediction
9. TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) - Program for displaying and printing phylogenies
10. WEBPHYLIP (http://sdmc.krdl.org.sg:8080/~lxzhang/phylip/) - It is a web version of the original PHYLIP package.

Bioinformatics Tools and URLs

EST Analysis

Name	URL
NCBI	http://www.ncbi.nlm.nih.gov/
Vector Screening	http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html
TrimEST	http://inn.weizmann.ac.il/cgi-bin/EMBOSS/emboss.pl?_action=input&_app=trimest
TrimSeq	http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=trimseq
RepeatMasker	http://repeatmasker.org
CAP3	http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=cap3
BLASTX	http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&BLAST_PROGRAMS=blastx&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome
ESTEXPLORER	http://estexplorer.els.mq.edu.au/estexplorer/main_page.php

Phylogenetic Analysis

Name	URL
ClustalW	http://www.ebi.ac.uk/Tools/clustalw2/index.html
MEGA5	http://www.megasoftware.net/

SSR identification

Name	URL
SSRprimerII	http://flora.acpfg.com.au/ssrprimer2/cgi-bin/index
Websat	http://wsmartins.net/websat/
MISA	http://pgrc.ipk-gatersleben.de/misa/

SNP identification

Name	URL
AutoSNP	Please contact Dave.Edwards@uq.edu.au

MicroRNA identification

Name	URL
miRBASE	http://www.mirbase.org/index.shtml
microcosm	http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/
miRSeek	http://220.227.138.213/mirnablast/mirnablast.php
psRNATarget	http://bioinfo3.noble.org/psRNATarget/
microinspector	http://bioinfo.uni-plovdiv.bg/microinspector/
mfold	http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1-2.3.cgi

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