Genetic diversity and differentiation of 50 Colletotrichum spp. isolates associated with leguminosae using multigene loci, RAPD and ISSR

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Genetic diversity and differentiation of 50 Colletotrichum spp. isolates from legume crops studied through multigene loci, RAPD and ISSR analysis. DNA sequence comparisons by six genes (ITS, ACT, Tub2, CHS-1, GAPDH, and HIS3) verified species identity of C. truncatum, C. dematium and C. gloeosporioides and identity C. capsici as a synonym of C. truncatum. Based on the matrix distance analysis of multigene sequences, the Colletotrichum species showed diverse degrees of intera and interspecific divergence (0.0 to 1.4%) and (15.5–19.9), respectively. A multilocus molecular phylogenetic analysis clustered Colletotrichum spp. isolates into 3 well-defined clades, representing three distinct species; C. truncatum, C. dematium and C. gloeosporioides. The ISSR and RAPD and cluster analysis exhibited a high degree of variability among different isolates and permitted the grouping of isolates of Colletotrichum spp. into three distinct clusters. Distinct populations of Colletotrichum spp. isolates were genetically in accordance with host specificity and inconsistent with geographical origins. The large population of C. truncatum showed greater amounts of genetic diversity than smaller populations of C. dematium and C. gloeosporioides species. Results of ISSR and RAPD markers were congruent, but the effective maker ratio and the number of private alleles were greater in ISSR markers.

Keywords: Colletotrichum spp., genetic diversity, ISSR, multigene loci, RAPD

Since the initial report of DNA amplification using PCR, the number of different applications of the technique has grown exponentially (Mullis et al., 1986). One of the first applications of PCR in mycology was described in 1990 by White and co-worker and dealt with the amplification and direct sequencing of ribosomal DNA (rDNA) to establish the taxonomic and phylogenetic relationships among fungi (White et al., 1990). The advent of PCR has enabled the development of powerful molecular makers for the detection or discrimination of fungi, either at the species or at the strain level, and extensive applications have largely been found in mycology, including taxonomy, phylogeny, and diagnostics. PCR-based detection of pathogenic fungi has been reported for several important genuses such as Phytophtora sp., Fusarium sp. and Colletotrichum sp. (Sherriff et al., 1994; Than et al., 2008; Cai et al., 2009; Cannon et al., 2012). PCR-based techniques based on DNA sequencing have the advantage of being specific, sensitive, and rapid compared to many other detection methods (Casimiro et al., 2004). A wealth of methodologies exists for detection of microorganisms, including traditional quantification of fruiting structures, scoring of disease symptoms, biochemical and microbiological methods. Recently, polymerase chain reaction (PCR)-based methods have gained an astonishing popularity in the field of diagnostics, because of sensitivity, specificity and ease of implementation (Sachse, 2004).

Ciampi et al. (2008) stated in the rating of genetic structures of pathogen populations it is good to know about their characteristic and evolutionary processes that shaped those populations in agroecosystems. Pathogen populations with a “mixed reproduction system, high gene flow, large effective population sizes, and high mutation rates” are more likely to overcome genetic resistance from hosts than populations with opposite characteristics (McDonald and Linde, 2002). Molecular analysis using RFLP, RAPD and ISSR demonstrated that occasional transfer of specific chromosomes may occur between apparently genetically distinct isolated clonal lines of biotypes of Colletotrichum spp. producing new pathotypes on different host plant (Masel et al., 1996). In addition to sexual recombination, diversity in fungal populations can also

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arise from transposable elements and mitotic reciprocal translocation events (Kistler and Miao, 1992). Genetic diversity can result from accumulation of mutations, and these mutational processes can produce variation within a species or population (Milgroom, 1996). In many instances constraints on genetic recombination such as mycelia or vegetative incompatibility exist in asexual pathogen populations producing clonal population structures (Masele et al., 1996). Molecular assays such as RAPD, ISSR markers and other techniques have elucidated genotype variation within and among these species. Such findings have led to more complex population species evaluations and to implications that members may in fact be subdivided into strains or isolate groups, based upon these markers. RAPD markers have become useful because this polymerase chain reaction (PCR) based technique is easy to implement by amplifying random DNA segments with short oligonucleotide primers (Williams et al., 1990). Primers with more than 50% GC content are desired because six or more GC base pair can form a strong enough duplex to be extended during polymerization. Guthrie et al. (1992) showed the potential use of RAPDs for identifying and differentiating isolates of Colletotrichum sp. that were collected from different regions of Africa, the United States, India and Puerto Rico. Results from this work indicated that some isolates have characteristic patterns based on geographic origin (Guthrie et al., 1992). RAPD also requires no sequence information, because a single short (10 base long) oligonucleotide of random sequence fulfills the same function as both primers in traditional PCR and there is a high probability of its annealing in many parts of the genome. When two primer binding sites on opposite strands fulfill certain criteria of proximity and orientation, the fragments will be multiplied exponentially. These criteria are not frequently fulfilled throughout the genome, but where the conditions are met, the DNA being amplified will tend to be the same set of fragments from related isolates (McDonald, 1997; Sherriff et al., 1995). RAPD markers have many complications that must be weighed against their relative ease of use, that make them difficult to reproduce between laboratories and sometimes within the same laboratory (McDonald, 1997). One explanation for different results from identical experiments is that RAPD pattern may be influenced by many technical factors that may be differ among laboratories (Penner et al., 1993). ISSR are hyper-variable markers based on microsatellites, but rather than genotyping microsatellite themselves, primers are designed to amplify the regions between microsatellite loci. This produces a genetic fingerprint from single primer polymerase chain reactions (Hettwer and Gerowitt, 2004; Mort et al., 2003; Wolfe et al., 1998). Legumes are the second most important crop plants after cereals in their importance based on area harvested and total production. They have been grown in about 190 million hectares, and the world’s production is approximately 300 million tons worldwide (Graham and Vance, 2003). The ability of legumes to fix atmospheric nitrogen (N) in the soil through their symbiotic association with Rhizobium bacteria, thereby reducing the need for fertilizers, and to serve as rotation crops is very important to current agricultural production systems (Serraj, 2004). There have only been a few dispersed studies on the causal agents of anthracnose disease on legumes in Malaysia. The literature search indicated that no thorough investigations have even been carried out on the causal agent of anthracnose on legumes in Malaysia. Therefore, studies on Colletotrichum species associated with anthracnose of legume crops in Malaysia have addressed in this research. In addition, the genetic diversity of Colletotrichum spp. populations from diverse host plants of legumes and different geographical region was investigated. The results of the present study provide better insights into the diversity and etiology of the pathogen and this will be helpful in the development of better disease management strategies and in the breeding of resistant varieties for legume production in Malaysia. The research focused on determining the genetic diversity among isolates of Colletotrichum spp. associated with grain legumes in Malaysia. Therefore, Multigene loci and molecular makers (RAPD and ISSR) were used to detect variations within and among species population.

Materials and Methods

Fungal isolates. Colletotrichum spp. isolates were obtained from symptomatic legume crops, including soybean, bean, pea, limabean, lentil, chickpea, peanut, cowpea, winged bean and country bean from various experimental and commercial farms in west of Malaysia, from the states of Selangor and Pahang (Table 1). Isolates collected from these locations, represented a range of geographic areas, host plants and species histories. Small pieces of plant tissues were taken out from the edge of healthy and infected tissues, surface sterilized in 1% sodium hypochlorite for 2 minutes, rinsed twice with distilled water, and after drying on sterilized tissue paper, plated on PDA (Potato Dextrose Agar). Where, spore masses were formed on plant tissues, the single spore isolation technique was applied for isolation. Conidia masses from lesions on the surface of the pods were removed with a sterile needle. A reasonable spore suspension was made with spore masses and spread onto