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Molecular Identification of the Entomopathogenic fungus, *Metarhizium anisopliae* isolate Released for Biological Control of Legume Flower Thrips, *Megalurothrips sjostedti* (Trybom)

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ABSTRACT

The control of insect pests with entomopathogens requires the ability to distinguish released pathogen from locally endemic isolates of the same species. In this study, random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique was carried out on *Metarhizium anisopliae* (Metsch.) Sorok. isolate ICIPE 69 (isolated from a soil sample) released for biological control of *Megalurothrips sjostedti* (Trybom) on cowpea in Kenya, the recovered isolate and five other isolates from different host and geographic locations. RAPD bands for the recovered isolate, ICIPE 69, was identical to those of the released isolate but different from other five isolates recovered from *Maruca vitrata* Fab. and *Clavigralla* spp collected from the same treated plot and other isolates obtained from the germplasm. This relatively easy, fast and efficient technique has proved useful in identification of entomopathogenic hyphomycete used for thrips control and may also be valuable in modelling epizootiology of the released pathogen.

INTRODUCTION

The global concerns of reducing chemical pesticide usage in agriculture due to negative ecological effects have provided strong impetus for the development of biological agents including entomopathogenic fungi for the control of pest insects (Lacey and Goettel, 1995). There is at present a world-wide resurgence of interest in the use of entomopathogenic fungi as biological control agents (BCA), and several of these agents have been or are in the process of being commercialized and significant improvement in development and manufacturing is expected with recent biotechnological innovations (Shah and Goettel, 1999). In Africa, one isolate of these agents of entomopathogenic fungi, *Metarhizium anisopliae* (Metsch.) Sorok. Has shown

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considerable promise in the management of thrips, including *Megalurothrips sjostedti* (Trybom), *Thrips tabaci* Lindeman and *Frankliniella occidentalis* (Pergande) on a variety of horticultural and floricultural crops (Ekesi *et al.*, 1998, 1999; Ekesi and Maniania; 2002; Tamò *et al.*, 2003). *Metarhizium anisopliae*, however, has a broad host range and have been recovered from over 300 insects and from soil samples (Moore and Prior, 1993). According to Bidochka (2001) an integral part of the measurement of success or failure of fungal microbial ecology must address the fate of the released fungus and ascertain whether it was the released pathogen that actually caused the desired effect. The accurate identification of a released BCA from those that already exist (i.e. locally endemic populations) within the agroecosystem is, therefore, crucial to the success of any biological control programme (Bidochka, 2001). The relatedness of various organisms in this manner, can genetically be assessed by various methods including isoenzyme analysis, restriction fragment length polymorphisms (RFLP) and a relatively new method, the random amplified polymorphic DNA analysis (RAPD). RAPD analysis uses a decanucleotide primer consisting of arbitrarily defined sequence in the polymerase chain reaction (PCR). The PCR is a highly efficient method of amplifying discrete DNA fragments using a thermostable DNA polymerase and single-stranded DNA primer (Mullis *et al.*, 1994). The DNA fragments that are generated in the RAPD reaction are then separated electrophoretically on an agarose gel and detected by ethidium bromide staining. The method can distinguish genetic diversity among individuals by separating the number and size of generated fragments using a single primer sequence. Generally individuals of the same genotype can be expected to have identical RAPD fragment profiles that are likely to differ from those of other genotypes thus providing finger-print-like information that can be used to compare individuals. In this study, the application of RAPD methodology is used to differentiate isolates of *M. anisopliae* released for the control of *M. sjostedti* from other locally endemic populations of the same species.

MATERIALS AND METHODS

Release and recovery of fungal isolates

During the short rains (October-December) of 2001, *M. anisopliae* isolate ICIPE 69 originating from a soil sample in the Democratic Republic of Congo (DRC) (Table 1) was applied as aqueous suspension of conidia (1×10^{13} conidia ha⁻¹) to an experimental cowpea (variety ICV 2) field (20 x 30 m) near the International Centre of Insect Physiology and Ecology (ICIPE) Headquarters, Nairobi, Kenya. Plant

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arrangement was 30 cm intra-row by 60 cm inter-row. The fungus was applied to the cowpea at 45 days after emergence (about 50% flowering and 25% podding). The plot was heavily infested with *M. sjostedti*, *Maruca vitrata* Fab., *Aphis craccivora* Koch and species of *Clavigralla* at the time of application. At 14 days after fungal application, one isolate of *M. anisopliae* was recovered from infected adult *M. sjostedti*. Similarly, at 21 days after treatment two isolates of *M. anisopliae* were recovered from larva of *M. vitrata* and nymph of *Clavigralla* sp. found in the treated plots. Three additional isolates from different hosts and geographical locations were also included in the analysis for comparison (Table 1). All isolates are at present accessioned as cryopreserved cultures at the ICIPE microbial bank.

TABLE 1. Origin of isolate of *Metarhizium anisopliae* used in the study

Isolate of isolation	Host (family)/substrate	Locality (country)	Date
ICIPE 17	<i>Maruca vitrata</i> F. (Pyralidae)	Mbita (Kenya)	2001
ICIPE 19	<i>Clavigralla</i> sp. (Coreidae)	Mbita (Kenya)	2001
ICIPE 24	<i>Busseola fusca</i> F. (Noctuidae)	Mbita (Kenya)	1989
ICIPE 25	Soil sample	Migori (Kenya)	1991
ICIPE 69	Soil sample	Kinshasa (DRC)*	1990
ICIPE 78	<i>Bemisia tabaci</i> G. (Aleyrodidae)	Kinshasa (DRC)*	1995

* DRC = Democratic Republic of Congo

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Monoconidial cultures of each of the fungal isolates were produced and maintained on sabouraud dextrose agar with yeast extract (SDY) i.e 40 g dextrose, 10 g mycological peptone, 10 g agar in 1 litre of distilled water, pH 5.6 at 26°C. Mycelia were obtained in submersed culture of spores in SDY broth excluding agar and incubated at room temperature with shaking at 150 rpm at 25°C for 3 days. The mycelium was harvested by filtration through filter paper (Whatman No.1) and then lyophilized and stored at - 80°C.

Extraction of DNA

Extraction of genomic DNA from the lyophilized mycelia followed the method of Tigano-Milani *et al.* (1995) with slight modifications. Briefly, approximately 30 mg of lyophilized mycelium was ground under liquid nitrogen to a powder in a 1.5 ml microfuge tube with a sterile toothpick. The powder was then suspended in 500 µl of sterile lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 100 mM EDTA, 10% SDS) and the tubes were vortexed. After 1 hr at 37°C, 75 µl of 5 mM NaCl was added and mixed, 60 µl of CTAB solution (10% CTAB in 7 mM NaCl) was added and mixed and the suspension was incubated at 65°C for 20 min. The supernatant was extracted with 1:1 buffered phenol:chloroform; ammonium acetate was added to a concentration of 2.5 M, and the mixture was extracted with chloroform. Nucleic acids were precipitated with 0.54 vol isopropanol, washed with 70% ethanol, and dried briefly in a heating block at 50°C. The purified DNA was resuspended in water and quantified fluorometrically and verified against DNA concentration standards using agarose gel electrophoresis.

Arbitrarily primed PCR

RAPD-PCR reactions were performed in a 20 µl volume with the following reaction mix: 10 ng genomic DNA; 10 mM KCl; 10 mM Tris-Cl, pH 8.3; 2.5 mM MgCl₂; 200 µM each Dntp; 0.5 µM primer; 2 units AmpliTaq DNA polymerase, Stoffel fragment (Perkin-Elmer Cetus) (Sobral and Honeycutt, 1993). The reactions were prepared on ice in 500 µl mineral oil (Perkin-Elmer Cetus), and placed in a System 9600 cycler (Perkin-Elmer). The temperature profile for all reactions was 94°C for 3 min, followed by 40 cycles of 94°C for 1 min, 35°C for 1 min, 72°C for 2.5 min, with a final extension at 72°C for 7 min (Sobral and Honeycutt, 1993). All reaction tubes, pipette tips, micropestles, and water (aliquoted in microcentrifuge tubes) were irradiated with UV light to destroy possible contaminants (Ou *et al.*, 1991). Irradiation treatment was 20 min at 2.5 cm from the bulb of a Genelinker (Biorad, Inc) UV light source. To further reduce the incidence of contamination, all reactions were assembled aseptically.

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in a laminar flow hood. Products were electrophoretically separated on 1.5% agarose gels, visualized with ethidium bromide and photographed.

Eight primers (Operon Technologies, Alameda, California) were tested on genomic DNAs of the *M. anisopliae* isolates (Table 2). Of these eight, OPA-07 was selected for this study based on its ability to produce easily distinguishable polymorphisms.

TABLE 2. Base-pair sequence of RAPD primers used in the study

OPA-04	AATCGGGCTG
OPA-06	GGTCCCTGAC
OPA-07	GAAACGGGTG
OPA-08	GTGACGTAGG
OPA-09	GGGTAACGCC
OPA-11	CAATCGCCGT
OPA-15	TTCCGAACCC
OPA-18	AGGTGACCGT

RESULTS AND DISCUSSION

RAPD profiles were observed for the 6 isolates tested in the study (Fig. 1). Isolate ICIPE 69, which originated from the soil sample in Democratic Republic of Congo (DRC) could clearly be distinguished from all other isolates. The recovered isolate collected from the infected cadaver of *M. sjostedti* at the experimental plot shared profiles that were identical with the released isolate but different from all other isolates tested (Fig. 1). Isolates ICIPE 17 and 19 from *M. vitrata*, and *Clavigralla* sp., respectively, which were obtained from the same experimental plot following spray application of ICIPE 69 had different profiles (Fig. 1). The presence of the 658-bp fragment and absence of 1242-bp distinguishes isolate ICIPE 78 from the

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released/recovered isolate ICIPÉ 69 (Fig. 1). However, given the close correspondence of its other profiles to those of ICIPÉ 69 and having been isolated from the same locality in 1995, it probably represent a descendant from ICIPÉ 69 attacking *Bemisia tabaci* G. in the DRC.

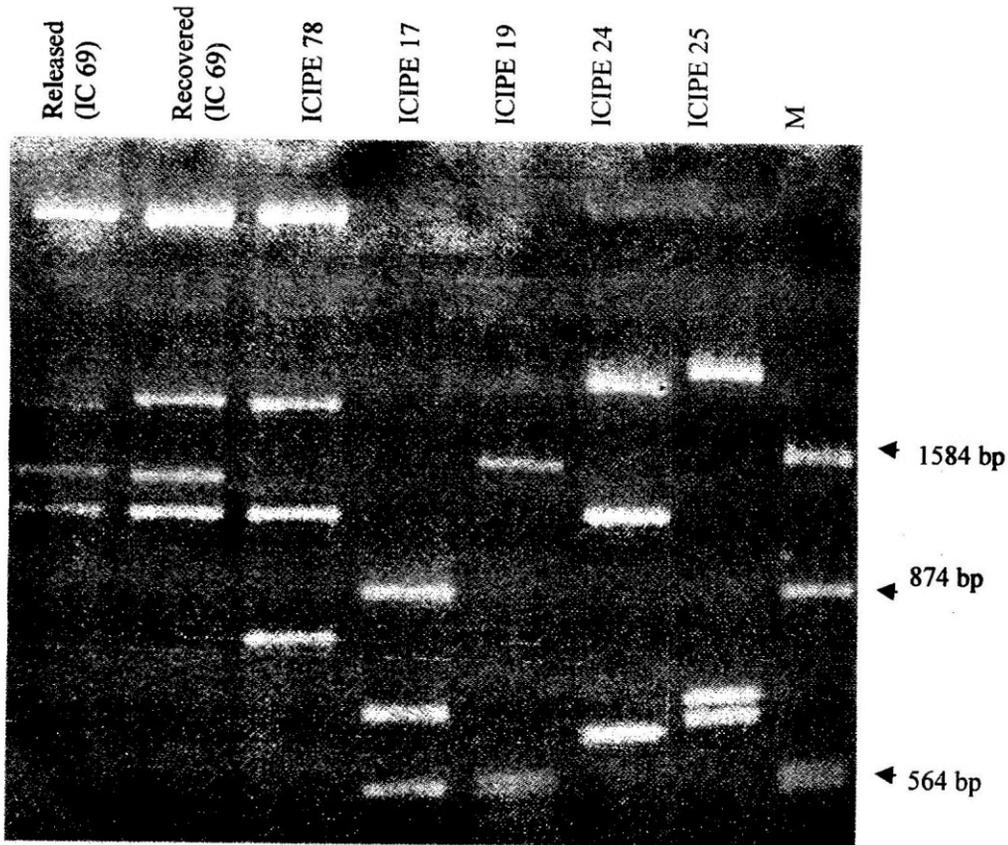


Fig. 1. RAPD bands of release and recovered isolate of ICIPÉ 69 compared with those of other isolates derived from other insects and soil substrate. Lane marked M denote molecular size standard

Understanding genetic variation and processes that derive genetic changes in pathogen populations can provide useful information in the development of control strategies (McDonald *et al.*, 1989). The data presented demonstrate the level of polymorphism present in *M. anisopliae* isolates. The six isolates tested produced different profiles. To assess the genetic variation that may exist within the isolates more accurately, the selected primer OPA-07 was initially tested with KlenTaq polymerase, AmpliTaq and AmpliTaq Stoffel fragment. However, apart from the *Taq* Stoffel fragment, the other enzymes produced erratic amplifications with many of the reactions amplifying only small amount of product, often with variable RAPD profiles or no product at all. This circumstance may be attributed to different activities of the enzyme or higher $MgCl_2$ concentration required for optimal Stoffel fragment activity. Generally,

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Stoffel fragments, which are genetically engineered deletion variants of thermostable polymerases have been reported to be more efficient in fingerprinting genomes than their native counterparts (Sobral and Honeycutt, 1993).

Numerous interacting factors affect genetic structure of a particular pathogen. Variations observed among pathogen populations are normally a reflection of the biology and the history of the species in different geographical areas (Milgroom and Lipari, 1995). All the isolates originated from different host and geographical locations and such variations may have contributed to the differences observed in the profile in this study. The existence of host-adapted lineages has been reported for entomopathogenic fungi especially within the entomophthorales (Papierok *et al.*, 1984; McGuire *et al.*, 1987; Glare, 1988). RAPD-PCR technique has been previously used for distinguishing isolates, strains and species of fungal pathogens. Within the fungi, with entomopathogenic properties, RAPD has been used to distinguish the varieties of *Paecilomyces fumosoroseus* (Wize) Smith & Brown, *Beauveria bassiana* (Bals.) Vuill. and in investigating intraspecific and interspecific variations in *Metarhizium* (Cobb and Clarkson, 1993; Fegan *et al.*, 1993; Bidochka *et al.*, 1994; Tigano-Milani *et al.*, 1995; Castrillo *et al.*, 1999).

The RAPD profiles of the released isolate were highly similar to the recovered isolate and provide additional information on the specificity of isolate ICIPE 69 to thrips and its success in the management of *M. sjostedti* in east and west Africa (Ekesi and Maniania, 2002; Tamò *et al.*, 2003). Using isoenzyme analysis, Milner and Mahon (1985) demonstrated that an Israeli isolate of *Zoophthora radicans* B. released in Australia became established in populations of the spotted alfalfa aphid and were able to distinguish the released isolate from an endemic strain of the fungus isolated from Diptera and Lepidoptera. Hodge *et al.* (1995) showed that RAPD fragment profiles of five recovered isolates of *Z. radicans* used for potato leafhopper control were very similar to those of the released isolates and different from all other isolates recorded from Lepidoptera. On the contrary, Silvie *et al.* (1990) using isoenzyme analysis were able to show that strains of *Pandora neoaphidis* H. released in a greenhouse experiment for the control of lettuce aphids were gradually displaced by indigenous strains of the fungus, which could be readily distinguished by this technique. Our results therefore highlight the importance of defining intra-species variability when designing strategies for monitoring releases of BCA into the environment and in subsequent recovery and positive identification of released isolates. The large numbers of reproducible bands generated by RAPD-PCR make this technique highly suited for such identification and most significantly the approach is fast, easy and quite efficient (Black, 1993). In future,

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this technique and the information generated here may also be useful in the development of epizootiological models, and improving our understanding of the population structure and persistence of isolate ICIPE 69 for the control of targeted thrips species.

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REFERENCES

- BIDOCHKA, M.J. 2001.** Monitoring the fate of biocontrol fungi. In: *Fungi as Biocontrol Agents: Progress, Problems and Potential* pp. 193-218 (ed. T.M. Butt, C.W. Jackson and N. Magan). CABI, Wallingford.
- BIDOCHKA, M.J., MCDONALD, M.A. ST. LEGER, R.J. and ROBERTS, D.W. 1994.** Differentiation of species and strains of entomopathogenic fungi by random amplification of polymorphic DNA (RAPD). *Current Genetics* 25: 107-113.
- BLACK, W.C. 1993.** PCR with arbitrary primers: approach with care. *Insect Molecular Biology* 2: 1-6.
- CASTRILLO, L.A., WIEGMANN, B.M. and BROOKS, W.M. 1999.** Genetic variation in *Beauveria bassiana* populations associated with the darkling beetle, *Alphitobius diaperinus*. *Journal of Invertebrate Pathology* 73: 269-275.
- COBB, B.D. and CLARKSON, J.M. 1993.** Detection of molecular variation in the insect pathogenic fungus, *Metarhizium* using RAPD-PCR. *FEMS Microbiology Letters* 12: 319-324.
- EKESI, S., MANIANIA, N.K., AMBONG-JUADIC, D. and OJIO, E. 1998.** Potential of the entomopathogenic fungus, *Metarhizium anisopliae* for the control of legume flower thrips, *Megalurothrips sjostedti* (Trybom) on cowpea in Kenya. *Crop Protection* 17: 661-668.

Genetic Identification of *Metarhizium anisopliae* isolates

- EKESI, S., MANIANIA, N.K., AMPONG-NYARKO, K. and ONU, I. 1999. Effect of intercropping cowpea with maize on the performance of *Metarhizium anisopliae* against the legume flower thrips, *Megalurothrips sjostedti* (Thysanoptera: Thripidae) and predators. *Environmental Entomology* 28: 1154-1161.
- EKESI, S. and MANIANIA, N.K. 2002. *Metarhizium anisopliae*: An effective biological control agent for the management of thrips in horti- and floricultural Crops in Africa. In: *Advances in Microbial Control of Insect Pests* pp. 145-165. (ed. R.K. Upadhyay). Kluwer Academic/Plenum Publishers, The Netherlands.
- LI PAN, M., MANNERS, J.M., MACLEAN, D.J., IRWIN, J.A.G., SAMUELS, K.D.Z., HOLDOM, D.G. and LI, D.P. 1993. Random amplified polymorphic DNA markers reveal a high degree of genetic diversity in the entomopathogenic fungus *Metarhizium anisopliae*. *Journal of General Microbiology* 139: 2075-2081.
- LEARE, T.R. 1988. Variation in the entomopathogenic fungal genus *Zoophthora*, with special reference to *Z. radicans*. Ph.D. dissertation, Australian National University. 121 pp.
- HODGE, K.T., SAWYER, A.J. and HUMBER, R.A. 1995. RAPD-PCR for identification of *Zoophthora* isolates in biological control of the potato leafhopper. *Journal of Invertebrate Pathology* 65: 1-9.
- LACEY, L.A. and GOETTEL, M.S. 1995. Current developments in microbial control of insect pests and prospects for the early 21st century. *Entomophaga* 40: 3-27.
- McGUIRE, M.R., MADDOX, J.V. and ARMBRUST, E.J. 1987. Host range studies of an *Erynia radicans* strain (Zygomycetes: Entomophthorales) isolated from *Empoasca fabae* (Homoptera: Cicadellidae). *Journal of Invertebrate Pathology* 50: 75-77.
- McDONALD, B.A., MCDERMOTT, J.M., GOODWIN, S.B. and ALLARD, R.W. 1989. The population biology of host-pathogen interactions. *Annual Review of Phytopathology* 27: 77-94.

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- MILGROOM, M.G. and LIPARI, S.E. 1995. Population differentiation in the chestnut blight fungus, *Cryphonectris parasitica*, in the eastern North America. *Phytopathology* 85: 155-160.
- MILNER, R.J. and MAHON, R.J. 1985. Strain variation in *Zoophthora radicans*, a pathogen on a variety of insect hosts in Australia. *Journal of Australian Entomological Society* 24: 195-198.
- MOORE, D. and PRIOR, C. 1993. The potential of mycoinsecticides. *Biocontrol News and Information* 14: 31N-40N
- MULLIS, K.B., FERRE, F. and GIBBS, R.A. 1994. *The Polymerase Chain Reaction*, Birkhauser, Boston.
- OU, C.Y., MOORE, J.L. and SCHOCHETMAN, G. 1991. Use of irradiation to reduce false positivity in polymerase chain reaction. *BioTechniques* 10: 442-445.
- PAPIERCK, B., TORRES, B.V.L. and ARNAULT, M. 1984. Contribution a l'étude de spécificité parasitaire du champignon entomopathogène *Zoophthora radicans* (Zygomycetes, Entomophthorales). *Entomophaga* 29: 109-119.
- SHAH, P.A. and GOETTEL, M.S. 1999. Directory of microbial control products and services. Microbial Control Division, Society for Invertebrate Pathology, Gainesville, FL, 36 pp.
- SILVIE, P., DEDRYVER, C.A. and TANGUY, S. 1990. Application expérimentale de mycelium d'*Erynia neoaphidis* (Zygomycetes: Entomophthorales) dans des populations de pucerons sur laitues en serre maraîchère: Étude du suivi de l'inoculum par caractérisation enzymatique. *Entomophaga* 35: 375-384.
- SOBRAL, B.W.S. and HONEYCUTT, R.J. 1993. High output genetic mapping in polypoids using PCR-generated markers. *Theory and Applications in Genetics* 86: 105-112.

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- TAMÒ, M., EKESI, S., MANIANIA, N.K. and CHERRY, A. 2003.** Biological control, a non-obvious component of integrated pest management for cowpea. In: *Biological Control in Integrated Pest Management Systems in Africa*, pp. 295-309 (ed P. Neuenschwander, C. Borgemeister & J. Langewald Eds.). CAB International, Wallingford.
- TIGANO-MILANI, M.S., HONEYCUTT, R.J., LACEY, L.A., ASSIS, R., MCCLELLAND, M. and SOBRAL, B.W.S. 1995.** Genetic variability of *Paecilomyces fumosoroseus* isolates revealed by molecular markers. *Journal of Invertebrate Pathology* 65: 274-282.