*Genetics and Molecular Biology*, 31, 1, 116-121 (2008)

www.sbg.org.br

Short Communication

# Sequence analysis of the rDNA intergenic spacer of *Metarhizium* strains isolated in Brazil

Fabiana Y. Yanaka-Schäfer[1], Leonara P. Dall'Onder[1], Mariana C. Panichi[1], Roberta G. Mendes[1], Nelson J.R. Fagundes[1,2], Josiane B. Bandinelli[1] and Maurício R. Bogo[1]

[1]*Laboratório de Biologia Genômica e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil.*
[2]*Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.*

## Abstract

To assess the extent of genetic variability of rDNA intergenic spacer (IGS) in *Metarhizium* sp., 34 strains (27 isolated in Brazil) were sequenced and analyzed together with an additional 20 *Metarhizium anisopliae* var. *anisopliae* sequences retrieved from GenBank. Overall, the global nucleotide diversity for the region under study was of 0.090, while for the Brazilian isolates it was only 0.016. Phylogenetic analyses showed four well-supported groups (A, B, C, and D), one of which (D) has not been previously identified. All but one of the Brazilian strains cluster in this novel D phylogroup, suggesting that the genetic variation found in Brazil is a subset of the worldwide *M. anisopiliae* var. *anisopliae* variation.

*Key words:* intergenic spacer, *Metarhizium*, rDNA.

Received: April 5, 2007; Accepted: July 24, 2007.

The entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin (Deuteromycotina, Hyphomycetes) is an "obligate killer" (Ebert and Weisser, 1997) which has consequently been much studied for potential use as a biological control agent of insect pests in many countries. The utilization and the improvement of mycoinsecticides could be an alternative tool to overcome the considerable environmental impact and high economical costs posed by conventional insecticides (Roberts and St. Leger, 2004). The non-transcribed intergenic region (IGS) and the internal transcribed spacers (ITS) located in the nuclear ribosomal DNA (rDNA) gene cluster evolve rapidly and have been widely used as a tool to evaluate the genetic diversity within populations of fungi (Mavridou and Typas, 1998; Pantou *et al.*, 2003; Hughes *et al.*, 2004; Velásquez *et al.*, 2007) and to resolve problems regarding the identification of species and isolates based on morphological characters (Milner *et al.*, 1994; Driver *et al.*, 2000; Iwen *et al.*, 2002).

Even though *Metarhizium* strains have been used in Brazil against a number of insect pests since the 1980s (Garcia *et al.*, 1984), an evaluation on the intraspecific genetic variability of *Metarhizium* isolates from Brazil has not so far been performed. Since better characterization of the genetic variation within an entomopathogenic fungus population could be useful for the selection of more efficient isolates for biological control, the aim of this study was to assess the extent of genetic variability in the IGS region of *Metarhizium* strains with special attention placed on the Brazilian isolates.

The 34 *Metarhizium* sp. isolates (27 isolated in Brazil) that were used in this study (Table 1) were provided by the following organizations: Brazilian Agricultural Research Corporation (Empresa Brasileira de Pesquisa Agropecuária - Embrapa) Genetics and Biotechnology Research unit (Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia - CENARGEN), Brasilia-DF, Brazil; School of Biological Sciences, University of Wales, University College Swansea, Swansea, Wales, UK; and Escola Superior de Agricultura Luiz de Queiroz (ESALQ) Brazil. The Isolates were cultivated in liquid complete Cove's medium (CCM) at 28 °C for 7 days and DNA was extracted and purified according to Raeder and Broda (1985). The IGS region of 34 *Metarhizium* strains was amplified using the Ma-28S4 and Ma-IGS1 primer set designed for amplification of *M. anisopliae* var. *anisopliae* rDNA (Pantou *et al.*, 2003). The PCR reactions contained 0.2 mM of dNTP,

Send correspondence to Maurício Reis Bogo. Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Av. Ipiranga 6681, Prédio 12C, Sala 172, 90619-900 Porto Alegre, RS, Brazil. E-mail: mbogo@pucrs.br.

**Table 1** - The *Metarhizium anisopliae* isolates characterized in this study, their hosts and geographic origins. Except where indicated all isolates were *Metarhizium anisopliae* var. *anisopliae.*

| *Metarhizium anisopliae* isolates* | GenBank number and host or source | Origin† |
|---|---|---|
| Brazilian isolates | | |
| From insects | | |
| CG34 | AY847509, *Conotrachelus* sp. (Coleoptera, Curculionidae) | AM |
| CG491 | AY847485, *Deois* sp. (Homoptera, Cercopidae) | PR |
| M5 | AY847491, *Deois* sp. (Homoptera, Cercopidae) | PE |
| CG31 | AY847499, *Deois flavopicta* (Homoptera, Cercopidae) | RJ |
| CG33 | AY847498, *Deois flavopicta* (Homoptera, Cercopidae) | MT |
| CG40 | AY847505, *Deois flavopicta* (Homoptera, Cercopidae) | DF |
| E6S2 | AY847483, *Deois flavopicta* (Homoptera, Cercopidae) | ES |
| CG153 | AY847503, *Deois incompleta* (Homoptera, Cercopidae) | PA |
| CG46 | AY847488, *Deois incompleta* (Homoptera, Cercopidae) | ES |
| CG143 | AY847484, Homoptera, Cercopidae | BA |
| V281 | AY847487, Homoptera, Cercopidae | Brazil |
| CG420 | AY847495, Hymenoptera | PB |
| CG858 | AY847504, *Mahanarva fimbriolata* (Homoptera, Cercopidae) | SP |
| CG28 | AY847492, *Mahanarva posticata* (Homoptera, Cercopidae) | AL |
| CG340 | AY847500, *Mahanarva posticata* (Homoptera, Cercopidae) | PE |
| CG626 | AY847508, *Mahanarva posticata* (Homoptera, Cercopidae) | AL |
| CG41 | AY847511, *Nezara viridula* (Hemiptera, Pentatomidae) | DF |
| CG144 | AY847490, *Piezodorus guildinii* (Hemiptera, Pentatomidae) | GO |
| CG423 (var. *acridum*) | AY847480, *Schistocerca pallens* (Orthoptera, Acrididae) | RN |
| CG43 | AY847502, *Zulia entreriana* (Homoptera, Cercopidae) | MG |
| CG578 | AY847507, *Zulia entreriana* (Homoptera, Cercopidae) | TO |
| From soil | | |
| CG37 | AY847497 | TO |
| CG39 | AY847493 | MS |
| CG347 | AY847494 | GO |
| CG393 | AY847512 | PR |
| CG419 | AY847501 | SE |
| CG489 | AY847510 | SP |
| Non-Brazilian isolates | | |
| V285 | AY847486, Coleoptera | Philippines |
| V288 | AY847477, Coleoptera | Philippines |
| CG515 | AY847496, *Nephotettix virescens* (Homoptera, Cicadellidae) | Philippines |
| CG644 (var. *majus*) | AY847478, *Oryctes* sp. (Coleoptera, Scarabaeidae) | Philippines |
| V90 | AY847479, *Sitona discoideus* (Coleoptera, Curculionidae) | France |
| Unknown provenance | | |
| E6S1 (sp. and var. unknown) | AY847481, Unknown | Unknown |
| V269 | AY847482, Unknown | Unknown |

*Source institutions: CG = Entomopathogenic Fungus Collection, EMBRAPA Recursos Genéticos e Biotecnologia, Brazil; V: School of Biological Sciences, Swansea, UK (Dr. Butt); strains E6S1, E6S2 and M5 were obtained from Escola Superior de Agricultura Luiz de Queiroz (ESALQ) Brazil.
†Brazilian state codes: AL, Alagoas; AM, Amazonas state; BA, Bahia; DF, Distrito Federal; ES, Espírito Santo; GO, Goiás; MT, Mato Grosso; MS, Mato Grosso do Sul; MG, Minas Gerais; PA, Pará; PR, Paraná; PB, Paraíba; PE, Pernambuco; RN, Rio Grande de Norte; RJ, Rio de Janeiro; SE, Sergipe; SP, São Paulo; TO, Tocantins.

0.4 μM of each primer, 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5 units of Taq DNA Polymerase (Invitrogen) and 2.0 mM of $MgCl_2$. Amplification used an initial denaturing step at 95 °C for 3 min followed by 30 cycles at 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min 30 s, followed by a final extension step at 72 °C for 10 min. The PCR products were purified with exonuclease I and shrimp alkaline phosphatase (GE Healthcare). The sequencing reactions were performed using the DYEnamic ET Dye Terminator Kit (GE Healthcare) and were read in a MegaBACE 1000

sequencer (GE Healthcare). After checking the chromatograms using the Chromas 2.23 program the DNA sequences were preliminarily aligned with ClustalX (Thompson *et al.*, 1997) and edited by visual inspection with the BioEdit program. An additional 20 strains of *M. anisopliae* var. *anisopliae* were retrieved from GenBank using BLAST (Table 2) and were also included in the analysis.

We selected the best evolutionary model in the Modeltest 3.06 program (Posada and Crandall, 1998), in which the K2P+G model (Kimura-2-Parameter with Gamma correction) received greatest support. Phylogenetic analysis using Neighbor-Joining (NJ) was performed with Mega 2.1 (Kumar *et al.*, 2001) based on K2P+G distance. To determine the reliability of the observed groups, 1000 bootstrap replicates were performed. Maximum Parsimony (MP) trees were constructed in PAUP* 4.0 (Swofford, 2002) using ten replicates of heuristic searches with random addition of sequences, and branch support was evaluated with 100 bootstrap replicates. Maximum likelihood (ML) trees were generated with PHYML (Guindon and Gascuel, 2003), based on a NJ starting tree and using the K2P+G model with parameters estimated during tree search. The BOOTPHYML program was used to perform 200 bootstrap replicates. Finally, Bayesian Inference (BI) was carried out using MRBAYES (Ronquist and Huelsenbeck, 2003) under K2P+G using 1,000,000 MCMC steps and sampling every 100 steps. The chains became stationary after 40,000 steps, so previous steps were discarded as burn-in. The estimation of the tree with the highest posterior probability was done through the majority-rule consensus of the remaining 9,700 trees. For each clade, nucleotide diversity ($p$) and its standard error were calculated using Mega 2.1 (Kumar *et al.*, 2001).

The amplicons generated from *Metarhizium* isolates using the Ma-28S4 and Ma-IGS1 primers resulted in sequences of about 800 bp. The variability in length of IGS amplicons observed among the isolates sampled in this

**Table 2** - The rDNA intergenic spacer rDNA sequences of *Metarhizium anisopliae* var. *anisopliae* isolates retrieved from GenBank, with their hosts and geographic origins.

| Strain* | GenBank number and host or source | Origin |
|---|---|---|
| From insects | | |
| IMI298061 | AF363465, *Brontispa longissima* (Coleoptera, Hispidae) | Papua New Guinea |
| KVL 275 | AF363462, *Cydia pomonella* (Lepidoptera, Torticidae) | Austria |
| V38 | AF363477, Dermaptera, Forriculidae | England |
| ITALY-12 | AF487272, Lepidoptera, Pyrallidae | Italy |
| KVL 130 | AF363459, Lepidoptera, Noctuidae | Denmark |
| ATHUM2920† | AF363463, *Melolontha melolontha* (Coleoptera, Scarabaeidae) | France |
| IMBST9601 | AF363468, *Melolontha melolontha* (Coleoptera, Scarabaeidae) | Austria |
| IMBST9602 | AF363469, *Melolontha melolontha* (Coleoptera, Scarabaeidae) | Austria |
| IMI152222 | AF363460, *Myllocerus discolor* (Coleoptera, Curculionidae) | India |
| ARSEF439 | AF363466, *Teleogryllus commodus* (Orthoptera, Gryllidae) | Australia |
| ARSEF440 | AF363467, *Teleogryllus commodus* (Orthoptera, Gryllidae) | Australia |
| IMI168777ii | AF363461, *Schistocerra gregaria* (Orthoptera, Acrididae) | Ethiopia |
| IMI298059 | AF363464, *Scapanes australis* (Coleoptera, Scarabaeidae) | Papua New Guinea |
| From soil | | |
| KVL 96-31 | AF363470 | Denmark |
| KVL 97-1 | AF363471 | Denmark |
| KVL 97125 | AF363472 | Denmark |
| V245 | AF363475 | Finland |
| V248 | AF363476 | Finland |
| Unknown provenance | | |
| V219 | AF363473, Unknown | Unknown |
| V242 | AF363474, Unknown | Unknown |

*Source institutions: ATHUM = University of Athens Fungal Collection, Athens Greece; ARSEF = US Department of Agriculture, Agriculture Research Service Entomopathogenic Fungus Collection, USDA-ARS; IMBST = Institut für Mikrobiologie, Leopold-Franzens Universität, Innsbruck, Austria; IMI = International Mycological Institute, Egham, UK; ITALY = L. Rovesti, Centro di studio dei Fitopharmaci, Bologna, Italy; KVL = Royal Veterinary and Agricultural University, Frederiksberg, Denmark; V = School of Biological Sciences, Swansea, UK (Dr. Butt).
†Strain ATHUM2920 is also known as CBS247.64 or MUCL 9646.

study has also been observed in other studies with *Metarhizium* strains (Mavridou and Typas, 1998; Pantou *et al.*, 2003) and other fungi in the Hypocreales (Mishra *et al.*, 2002). Including all insertion sites, we obtained a final aligned data set of 983 bp. Insertion/deletion events were treated as missing data and therefore the phylogenetic analyses were performed based on 512 bp. The final alignment of the IGS sequences showed high nucleotide diversity among isolates ($\pi = 0.090 \pm 0.007$).

Because all methods resulted in trees with similar topologies, we only show the ML tree with the branch support values obtained from all methods (Figure 1). All methods showed the formation of four well-supported groups (A, B, C and D). Groups A, B and C were reported (with identical names) in other studies (Hughes *et al.*, 2004; Pantou *et al.*, 2003) and Hughes *et al.* (2004) also demonstrated that group A was subdivided in three subgroups (A1, A2 and A3). Our results support the existence of groups A, B and C, with the addition of a novel group (D), all with very high support values.

Group A showed a nucleotide diversity of $0.010 \pm 0.002$ and was composed of isolates IMBST9602, ATHUM 2920, KVL130, ARSEF439, ITALY12, IMI298059, KVL971 and CG393. In spite of all isolates being *M. anisopliae* var. *anisopliae*, they were from hosts belonging to different insect orders and from different geographic origins. Group A also contained the only Brazilian isolate that did not cluster in group D (CG393).

Group B isolates had a nucleotide diversity of $0.027 \pm 0.004$, shared a deletion at position 729-732 and clustered with *M. anisopliae* var. *anisopliae* isolates from European climates and collected from either insects or soil. Isolates V245, V218, KVL97125 and KVL9631 were from soil. Strains V219 and V242 were isolated from unknown hosts, but grouped consistently with isolates whose hosts belonged to the orders Dermaptera and Lepidoptera respectively. Although this could be an indication that these isolates came from insects, it is not possible to be very certain given that the sample size for this group was not large.

Group C was exclusively formed by isolates from Coleopteran hosts and was the group with the largest nucleotide diversity ($\pi = 0.054 \pm 0.006$). This group was characterized by the presence of three deletions (positions 217-229, 262-337 and 663-666) plus the 729 to 732 deletion also observed in Group B, and is the same grouping observed by Hughes *et al.* (2004) and Pantou *et al.* (2003) with the addition of isolates V90, CG644 and V288 from our study. Isolates CG644 and V288 were previously classified as *M. anisopliae* var. *majus*, but grouped consistently with other isolates classified as var. *anisopliae*. Given the difficulties that can arise from a morphology based classification (Milner *et al.*, 1994) we think that the more likely explanation is that these isolates were previously misidentified. Alternative explanations would imply that the *M. anisopliae* var. *anisopliae* isolates in this group were mis-

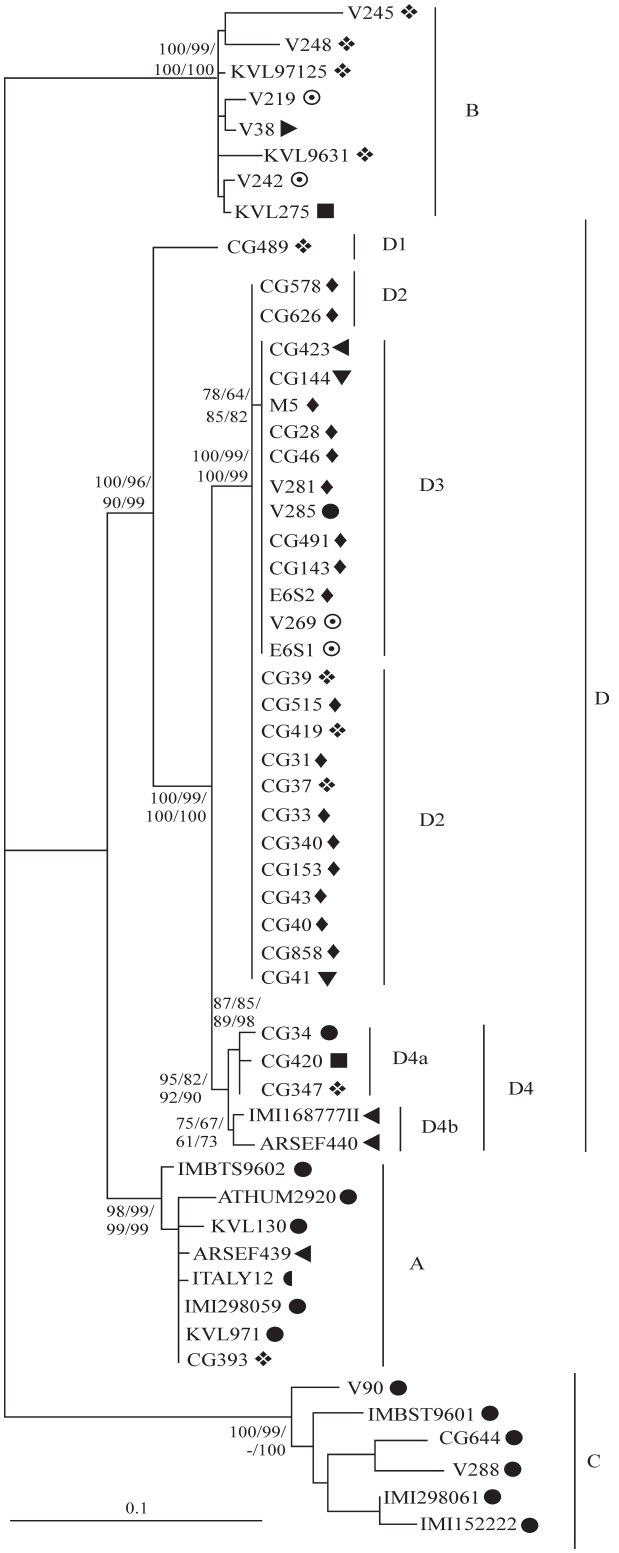


**Figure 1** - Phylogram of partial nucleotide sequence of the rDNA intergenic spacer (IGS) region in *Metarhizium* sp. based on maximum likelihood (ML) analysis. The numbers close to some branches represent the support values obtained from ML, number joining, maximum parsimony or Bayesian inference respectively. For clarity, we only present those values related to the groups discussed in the text. Symbols indicate the environment or host from which the isolate was obtained: ❖ Soil; ● Coleoptera; ▶ Dermaptera; ▼ Hemiptera; ◆ Homoptera; ■ Hymenoptera; ◖ Lepidoptera; ◀ Orthoptera; ⦿ unknown host.

identified or that IGS does not have enough power to discriminate between these varieties.

Group D, previously unidentified, included all but one of the Brazilian isolates together with other tropical isolates from Papua New Guinea, the Philippines, Australia and Ethiopia. This group had a nucleotide diversity of $\pi = 0.012 \pm 0.002$ and was divided into four subgroups D1, D2, D3 and D4, with subgroup D4 being further subdivided into D4a and D4b. Subgroup D1 contained only isolate CG489, which also presented one exclusive deletion (position 281-302). Some discrepancies about morphological and genetic species classification were found in subgroups D2 and D3. Our study strongly suggests that isolates CG423 (*M. anisopliae* var. *acridum*), V285 (*M. anisopliae* var. *majus*) and CG515 (*M. album*) are in fact all *M. anisopliae* var. *anisopliae*. This result was in agreement with former studies that demonstrated the lack of accuracy of morphological classification (Driver *et al.*, 2000). Isolates from subgroup D4a shared a common deletion at 379-564 bp, while isolates of D4b group had one deletion at 92-125 bp. Interestingly, isolates from group D4b were both isolated from an Orthoptera host, even though in different continents. On the other hand, isolates in group D4a were all from the same country but each one isolated from hosts belonging to different Insect orders.

Overall, the nucleotide diversity in the Brazilian isolates was $0.016 \pm 0.002$, considerably lower than the estimate of $0.124 \pm 0.008$ obtained when excluding the Brazilian isolates. Together with the finding that all but one of the Brazilian isolates belongs to Group D, this suggests that the genetic diversity of *M. anisopliae* var. *anisopliae* found in Brazil represents only a subset of the worldwide diversity within this variety.

Our results suggest that the IGS region can easily assign *M. anisopliae* var. *anisopliae* isolates to one of the existing clades, and that it may help to solve discrepancies in the current classification of these isolates, even though the number of strains with identical genetic profiles (Figure 1) suggests that it is not well suited for individual isolate identification. While to some extent it was possible to associate phylogenetic position of the isolates with the hosts from which they were isolated or to the climatic zone of origin, on a finer scale a strong association between genetic and host or geographic origin was not found. Finally, we observed that although almost all Brazilian isolates can be assigned to Group D there is some genetic diversity among Brazilian strains, even among samples isolated in the same Brazilian region.

## Acknowledgments

The authors thank the Brazilian state agency Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) for financial support and Brazilian Agricultural Research Corporation (Empresa Brasileira de Pesquisa Agropecuária - Embrapa) for providing the Brazilian strains. MCP was a recipient of a FAPERGS fellowship. RGM was a recipient of fellowship from the Catholic University Rio Grande do Sul (Pontifícia Universidade Católica do Rio Grande do Sul - BPA/PUCRS). The authors thank Dr. Soraya C.M. Leal-Bertioli for some DNA strains used in this work and Dr. Eduardo Eizirik for a critical reading of the manuscript. We also thank Cladinara R. Sarturi for technical laboratory assistance and Felipe G. Grazziotin for helpful discussion regarding the phylogenetic analysis.

## References

Driver F, Milner RJ and Trueman WH (2000) A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. Mycol Res 104:134-150.

Ebert D and Weisser WW (1997) Optimal killing for obligate killers: The evolution of life histories and virulence of semelparous parasites. Proc R Soc Lond 264:985-991.

Garcia AS, Messias CL, Souza HML and Piedrabuena AE (1984) Patogenicidade de *Metarhizium anisopliae* var. *anisopliae* em *Ceratitis capitata* (Wied.) (Diptera, Tephritidae). Rev Bras Entomol 28:421-424.

Guindon S and Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by Maximum Likelihood. Syst Biol 52:696-704.

Hughes WOH, Thomsen L, Eilenberg J and Boomsma JJ (2004) Diversity of entomopathogenic fungi near leaf-cuting and nests in a neotropical forest, with particular reference to *Metarhizium anisopliae* var. *anisopliae*. J Invertebr Pathol 85:46-53.

Iwen PC, Hinrichs SH and Rupp ME (2002) Utilization of the internal transcrib 17ed spacer regions as molecular targets to detect and identify human fungal pathogens. Med Mycol 40:87-109.

Kumar S, Tamura K, Jakobsen IB and Nei M (2001) MEGA2: Molecular Evolutionary Genetics Analysis software. Bioinformatics 17:1244-1245.

Velásquez VB, Cárcamo MP, Meriño CR, Iglesias AF and Durán JF (2007) Intraspecific differentiation of Chilean isolates of the enthomopathogenic fungi *Metarhizium anisopliae* var. *anisopliae* as revealed by RAPD, SSR and ITS markers. Gen Mol Biol 30:89-99.

Mavridou A and Typas MA (1998) Intraspecific polymorphism in *Metarhizium anisopliae* var. *anisopliae* reveled by analysis of rRNA gene complex amd mtDNA RFLPs. Mycol Res 102:1233-1241.

Milner RJ, Driver F, Curran J, Glare TR, Prior C, Bridge PD and Zimmermann G (1994) Recent problems with the taxonomy of the genus *Metarhizium*, and a possible solution. Proceedings of the VIth International Colloquium of Invertebrate Pathology and Microbial Control. Montpellier, pp 101-116.

Mishra PK, Fox RTV and Culham A (2002) Restriction analysis of PCR amplified nrDNA regions revealed intraspecific variation within populations of *Fusarium culmorum*. Microbiol Lett 215:291-296.

Pantou MP, Mavridou A and Typas MA (2003) IGS sequence variation, group-I introns and the complete nuclear ribosomal DNA of the entomopathogenic fungus *Metarhizium*:

Excellent tools for isolate detection and phylogenetic analysis. Fungal Genet Biol 38:159-174.

Posada D and Crandall KA (1998) Modeltest: Testing the model of DNA substitution. Bioinformatics 14:817-818.

Raeder U and Broda P (1985) Rapid preparation of DNA from filamentous fungi. Lett Appl Microbiol 1:17-20.

Roberts DW and St Leger RJ (2004) *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: Mycological aspects. Adv Appl Microbiol 54:1-70.

Ronquist F and Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572-1574.

Swofford DL (2002) PAUP*: Phylogenetic Analysis Using Parsimony (and Other Methods) 4.0 Beta, Florida State University. CD-ROM.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F and Higgins DG (1997) The ClustalX windows interface: Flexible strategies for multiple seqeunce alignment aided by quality analysis tools. Nuc Acids Res 24:4876-4882.

## Internet Resources

Chromas 2.23 at http://www.technelysium.com.au/chromas.html.

BioEdit program at http://www.mbio.ncsu.edu/BioEdit/bioedit.html.

*Associate Editor: Luis Carlos de Souza Ferreira*