Available online at www.sciencedirect.com
ScienceDirect
Journal of Invertebrate Pathology 93 (2006) 11–21
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Phylogenetic origins of African and Neotropical *Beauveria bassiana s.l.* pathogens of the coffee berry borer, *Hypothenemus hampei*

Stephen A. Rehner [a,*], Francisco Posada [a], Ellen P. Buckley [a], Francisco Infante [b], Alfredo Castillo [b], Fernando E. Vega [a]

[a] *Insect Biocontrol Laboratory, USDA, ARS, BARC-West, Beltsville, MD 20705-2350, USA*
[b] *El Colegio de la Frontera Sur (ECOSUR), Carretera Antiguo Aeropuerto km 2.5, Tapachula, 30700 Chiapas, Mexico*

Received 21 September 2005; accepted 18 April 2006
Available online 27 June 2006

Abstract

A phylogenetic epidemiological study of *Beauveria bassiana* s.l. was conducted for African and Neotropical pathogens of the coffee berry borer (CBB), *Hypothenemus hampei*, based on inferences from two nuclear intergenic regions, EFutr and Bloc. CBB pathogens were distributed among four terminal clades, however, the majority of African and Neotropical isolates cluster in a well-supported monophyletic group, informally designated AFNEO_1. Although the relationship between African and Neotropical AFNEO_1 is unresolved, the majority of alleles detected were exclusive to either the African or the Neotropical populations. These fixed genetic differences suggest that their disjunction predates the world trade in coffee. Neotropical AFNEO_1 have a broad host range and CBB pathogens are intermixed phylogenetically with isolates from diverse indigenous insects. Several Neotropical AFNEO_1 isolates were isolated from coffee plants as epiphytes or endophytes, thus plants themselves may potentially serve as reservoirs of pathogens against their insect pests. Topological incongruence between the EFutr and Bloc phylogenies of Neotropical AFNEO_1 may signify that individuals within this population are recombining.
Published by Elsevier Inc.

Keywords: *Beauveria bassiana*; *Hypothenemus hampei*; Coffee berry borer; Coffee; Biogeography; Phylogeny; EF1-α; Nuclear intergenic regions; EFutr; Bloc

1. Introduction

The coffee berry borer (CBB), *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae), is the most devastating pest of coffee beans worldwide. Endemic to Central Africa (Le Pelley, 1968), the range of CBB has expanded greatly over the last 100 years as a result of the world trade in coffee. *H. hampei* was first reported in Gabon in 1901 (Beille, 1925), in Java in 1908 (Hagedorn, 1910), and in Brazil in 1913 (de Oliveria Filho, 1927). The pest has continued to spread and now occurs in the majority of coffee producing regions throughout the world, and threatens the economic viability of coffee growing in many of these areas.

The biology of CBB presents formidable challenges for the implementation of pest management programs. Inseminated adult females bore into a coffee berry and lay their eggs in the endosperm, on which the larvae feed. Prior to emergence from their natal berry, adult females mate with male siblings and are thereby inseminated and ready to colonize another berry. In contrast, males are apterous and never leave the berry (Le Pelley, 1968; Bustillo et al., 1998). The insects therefore spend the majority of their life cycles cloistered within coffee berries, presenting a very limited time window during which females are exposed to the environment. AFLP fingerprinting of DNA from coffee berry borers collected in 17 countries has revealed an extremely low genetic variability in this species, most likely due to inbreeding and possible genetic bottlenecks occurring during the course of its geographic dispersion (Benavides et al., 2005).

* Corresponding author. Fax: +1 301 504 5104.
E-mail address: rehners@ba.ars.usda.gov (S.A. Rehner).

0022-2011/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.jip.2006.04.005

The use of conventional insecticides for the control of coffee berry borers is a very limited option due to the economic situation of small holder coffee growers, the potential adverse effects of pesticides to human health and the environment, and the development of pesticide resistance by the coffee berry borer to endosulfan (Brun et al., 1989; ffrench-Constant et al., 1994). These constraints have prompted research on biological control, including the introduction of parasitoids and fungal entomopathogens (see Bustillo et al., 1998; and reviews by Moore and Prior, 1988; Waterhouse and Norris, 1989; Barrera et al., 1990; Moore et al., 1990; Murphy and Moore, 1990; Damon, 2000), and the search for new pathogens (Vega and Mercadier, 1998; Vega et al., 1999, 2000).

Forty different fungal species in 22 genera have been found associated with CBB (Pérez et al., 2003) and various entomopathogens are known to infect the insect (Fernãndez et al., 1985; Balakrishnan et al., 1995; Bustillo et al., 1998; Posada-Flórez et al., 1998). Among entomopathogenic fungi associated with CBB, *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) is by far the most widely reported: Brazil (Averna-Saccá, 1930), Cameroon (Pascalet, 1939), Colombia (Vélez-Arango and Benavides-Gómez, 1990), Democratic Republic of the Congo (Steyaert, 1936), Ecuador (Klein-Koch et al., 1988), Guatemala (Monterroso, 1981), Honduras (Lazo, 1990), India (Balakrishnan et al., 1994), Indonesia (Friederichs and Bally, 1923), Mexico (Méndez-López, 1990), Nicaragua (Barrios, 1992), and Venezuela (Bautista, 2000).

As the principal fungal pathogen of CBB in all coffee growing regions, *B. bassiana* is a leading candidate for its biological control (Bustillo et al., 1998). However, little is known about the geographic or genetic origins of *B. bassiana* that are pathogenic to CBB. It is not known whether ancestral African *B. bassiana* pathogens co-dispersed from Africa with CBB during the global expansion of coffee agriculture or whether these insects have acquired *B. bassiana* pathogens indigenous to regions where CBB has been introduced. According to Bridge et al. (1990), allozyme data support a close relationship among the majority of 16 isolates of *B. bassiana* from CBB from ten countries (Brazil, Ecuador, Guatemala, Indonesia, Jamaica, Kenya, Mexico, New Caledonia, Sri Lanka, and Togo). Although it is possible that the inferred similarity among these *B. bassiana* isolates is due to a common, possibly African origin, the sensitivity and utility of isozymes as molecular markers in *Beauveria* is not well developed and has been superceded by nucleic acid characters. In a geographic survey of *B. bassiana* isolates from CBB and other insects, Gaitan et al. (2002) reported high variability in RAPD banding patterns, low polymorphism in ITS RFLP but no congruence between the groupings resolved with either type of marker. Genetic surveys of *B. bassiana s.l.* from other insects and geographic regions employing dominant markers (Maurer et al., 1997; Glare and Inwood, 1998; Aquino de Muro et al., 2003, 2005) or ITS-RFLP (Coates et al., 2002) also perform poorly in resolving genetic groupings informative to hypotheses of host selection or geographic speciation. Thus, neither RAPD, AFLP nor ITS has proven effective for delineating genetic boundaries or for inferring population genetic or epidemiological processes within *B. bassiana*.

In a recent molecular phylogenetic analysis of *Beauveria*, Rehner and Buckley (2005) showed that the morphospecies *B. bassiana* consists of two unrelated and morphologically indistinguishable clades. These clades are not yet recognized taxonomically, and we refer to them as the "*B. bassiana s.l.*" and the "pseudobassiana" clades. The former is a globally distributed group, believed to include the authentic *B. bassiana*, and the latter is an ad hoc designation for the alternate clade, which remains to be described. This investigation is concerned only with isolates derived within the *B. bassiana s.l.* clade.

In this study, we investigated the phylogenetic diversity of *B. bassiana s.l.* pathogens of CBB from Africa (Cameroon, Côte d'Ivoire, Kenya, Togo) and the Neotropics (Brazil, Colombia, Costa Rica, Mexico, Nicaragua). Phylogenetic analyses presented here are based on nucleotide variation at two intergenic regions, EFutr and Bloc, genetic markers developed expressly for analysis within the *B. bassiana s.l.* complex (SAR, unpublished). A broad geographic sampling of *B. bassiana s.l.* isolates was used to provide a genealogical context for inference of the phylogenetic origins and relationships of *B. bassiana s.l.* pathogens of CBB. Additionally, the relationships of Neotropical *B. bassiana s.l.* CBB pathogens to other co-occurring *B. bassiana s.l.* pathogens of indigenous insect species, and to isolates associated with coffee plants as epiphytes and endophytes, were also determined.

2. Materials and methods

2.1. Source of fungal isolates

Fungal isolates were obtained from the ARS Collection of Entomopathogenic Fungal Cultures (ARSEF; Ithaca, NY), the European Biological Control Laboratory (EBCL; Montpelier, France) or are maintained in the laboratory of FEV in the Insect Biocontrol Laboratory (IBL; Beltsville, MD). Collection data for the 75 *B. bassiana s.l.* isolates included in the phylogenetic analyses conducted in this study are listed in Table 1. The African isolates, all of which were isolated from CBB, included 57 isolates from Cameroon (9), Côte d'Ivoire (17), Togo (28) and Kenya (3). However, only six of these isolates, which encompass the allelic diversity detected in Africa, were included in the present analysis. The 45 Neotropical isolates originated from Brazil (13), Colombia (10), Costa Rica (1), Nicaragua (3), Mexico (18), and included isolates from CBB (29), indigenous Neotropical insects (13) and endophytes (3) and epiphytes of coffee (1). An additional 22 isolates from Asia (9), Australia (2), Europe (4), North America (5) and North Africa (2) representing lineages other than those associated with CBB were included to provide phylogenetic context. An isolate of *Beauveria brongniartii* (Sacc.) Petch, ARSEF 1041, was used as outgroup for some analyses.

Table 1
Beauveria bassiana s.l. isolates used in this study and EFutr and Bloc GenBank Accession Nos

Collection#[a]	Country	Host/substrate	Host Order: Family	EFutr	Bloc
ARSEF 32	USA	NA	Orthoptera: Acrididae	AY531930	DQ384407
ARSEF 300	Australia	*Nysius vinitor*	Hemiptera: Lygaeidae	AY531924	DQ384410
ARSEF 319	Mexico	NA	Coleoptera: Cerambycidae	DQ380093	DQ384381
ARSEF 326	Australia	*Chilo plejadellus*	Lepidoptera: Pyralidae	AY531929	DQ384411
ARSEF 344	USA	*Leptinotarsa decemlineata*	Coleoptera: Chrysomelidae	AY531932	DQ384413
ARSEF 652	China	*Ostrinia* sp.	Lepidoptera: Pyralidae	AY531941	DQ384391
ARSEF 656	PR China	*Nephotettix bipunctata*	Homoptera: Cicadellidae	AY531942	DQ384405
ARSEF 678	PR China	*Nephotettix bipunctata*	Homoptera: Cicadellidae	AY531946	DQ384406
ARSEF 681	Romania	*Leptinotarsa decemlineata*	Coleoptera: Chrysomelidae	AY531947	DQ384418
ARSEF 751	Vietnam	NA	Coleoptera: Chrysomelidae	AY531954	DQ384404
ARSEF 753	Brazil	*Aracanthus sp.*	Coleoptera: Curculionidae	AY531955	DQ384398
ARSEF 792	USA	*Blissus leucopterus*	Hemiptera: Lygaeidae	AY531957	DQ384408
ARSEF 1007	Japan	*Bombyx mori*	Lepidoptera: Bombycidae	DQ380138	DQ384395
ARSEF 1053	Brazil	NA	Orthoptera: Acrididae	AY531883	DQ384396
ARSEF 1153	Morocco	*Sitona discoideus*	Coleoptera: Curculionidae	AY531884	DQ384399
ARSEF 1398	France	*Hypera postica*	Coleoptera: Curculionidae	AY531888	DQ384401
ARSEF 1456	Brazil	*Diabrotica speciosa*	Coleoptera: Chrysomelidae	DQ380122	DQ384356
ARSEF 1480	Brazil	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380118	DQ384347
ARSEF 1628	Hungary	NA	Lepidoptera: Noctuidae	AY531896	DQ384400
ARSEF 1679	Japan	*Monochamus alternatus*	Coleoptera: Cerambycidae	AY531898	DQ384392
ARSEF 1811	Morocco	*Sitona discoideus*	Coleoptera: Curculionidae	AY531901	DQ384417
ARSEF 1829	Brazil	*Castnia licus*	Lepidoptera: Castniidae	AY531902	DQ384386
ARSEF 1831	Brazil	*Atta* sp.	Hymenoptera: Formicidae	DQ380095	DQ384380
ARSEF 1959	Brazil	NA	Orthoptera: Acrididae	AY531906	DQ384397
ARSEF 1966	Brazil	*Anthonomus grandis*	Coleoptera: Curculionidae	DQ380098	DQ384384
ARSEF 2040	Korea	NA	Coleoptera: Curculionidae	AY531910	DQ384389
ARSEF 2637	Brazil	NA	Lepidoptera larva, sp. indet.	DQ380112	DQ384354
ARSEF 2685	Kenya	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380107	DQ384349
ARSEF 2686	Kenya	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380135	DQ384390
ARSEF 2691	Togo	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380117	DQ384346
ARSEF 2692	Togo	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380116	DQ384345
ARSEF 2693	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380090	DQ384375
ARSEF 2722	Brazil	*Solenopsis* sp.	Hymenoptera: Formicidae	DQ380119	DQ384348
ARSEF 2883	USA	*Schizaphis graminum*	Homoptera: Aphididae	AY531919	DQ384412
ARSEF 3018	Brazil	*Forficula auricularia*	Dermaptera: Forficulidae	DQ380102	DQ384388
ARSEF 3097	USA	*Anthonomus grandis*	Coleoptera: Curculionidae	AY531925	DQ384409
ARSEF 3440	Nicaragua	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380096	DQ384382
ARSEF 3445	Nicaragua	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380120	DQ384357
ARSEF 3448	Nicaragua	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380097	DQ384383
ARSEF 3456	Costa Rica	NA	Hemiptera: Miridae	DQ380108	DQ384350
ARSEF 3818	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380091	DQ384376
ARSEF 3819	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380103	DQ384366
ARSEF 3999	Brazil	*Frankliniella occidentalis*	Thysanoptera: Thripidae	DQ380094	DQ384385
ARSEF 4093	Brazil	*Nezara viridula*	Hemiptera: Pentatomidae	DQ380101	DQ384387
ARSEF 4393	Japan	Soil isolate	NA	DQ380136	DQ384393
ARSEF 5438	Ivory Coast	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380115	DQ384344
ARSEF 5446	Togo	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380114	DQ384343
ARSEF 7273	Nepal	NA	Hemiptera: NA	DQ380130	DQ384402
ARSEF 7274	Nepal	NA	Lepidoptera: NA	DQ380131	DQ384403
Bb 9102	Colombia	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380085	DQ384370
Bb 9201	Colombia	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380137	DQ384394
Bb9205	Colombia	*Diatraea saccharalis*	Lepidoptera: Pyralidae	DQ380105	DQ384368
Bb 9305	Colombia	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380104	DQ384367
Bb 9417	Colombia	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380086	DQ384371
EBCL 99043	Cameroon	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380129	DQ384365
IBL 03005	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380123	DQ384359
IBL 03006	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380121	DQ384358
IBL 03010	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380100	DQ384377
IBL 03013	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380124	DQ384360
IBL 03015	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380126	DQ384362
IBL 03016	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380132	DQ384415
IBL 03017	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380092	DQ384379
IBL 03021	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380128	DQ384364

(*continued on next page*)

Table 1 (*continued*)

Collection#[a]	Country	Host/substrate	Host Order: Family	EFutr	Bloc
IBL 03022	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380134	DQ384414
IBL 03023	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380127	DQ384363
IBL 03024	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380125	DQ384361
IBL 03025	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380109	DQ384351
IBL 03026	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380099	DQ384353
IBL 03028	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380133	DQ384416
IBL 03029	Mexico	Hypothenemus hampei	Coleoptera: Curculionidae	DQ380110	DQ384352
IBL 03030	Colombia	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380113	DQ384355
IBL 03031	Colombia	*Coffea arabica*[a]	NA	DQ380087	DQ384372
IBL 03032	Colombia	*Coffea arabica*[b]	NA	DQ380088	DQ384373
IBL 03033	Colombia	*Coffea arabica*[b]	NA	DQ380089	DQ384374
IBL 03034	Colombia	*Prorops nasuta*	Hymenoptera: Bethylidae	DQ380106	DQ384369
IBL 03027	Mexico	*Hypothenemus hampei*	Coleoptera: Curculionidae	DQ380111	DQ384378

Collection codes: ARSEF, ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY; Bb, Cenicafé Entomopathogen Collection, Chinchiná, Caldas, Colombia; EBCL, European Biological Control Laboratory, Montpellier, France; IBL, Insect Biocontrol Laboratory, USDA, ARS, Beltsville, MD.

[a] Leaf epiphyte.

[b] Fruit endophyte.

2.2. DNA extraction

Isolates were grown as mycelia in quarter-strength SDY nutrient broth (Goettel and Inglis, 1997) for 3–5 days at 125 rpm, 25 °C, on an orbital shaker. The mycelia were collected by centrifugation, rinsed with sterile distilled water, frozen at −80 °C, and lyophilized and ground to a powder using a FastPrep tissue homogenizer (MP Biomedicals, Irvine, CA). Approximately 30 mg of the ground mycelium was suspended in 900 µl lysis buffer (2 M NaCl, 0.4% w/v deoxycholic acid, and 1.0% w/v polyoxyethylene ether) and incubated at 55 °C for 10 min. The cellular lysate was extracted with 750 µl chloroform:isoamyl alcohol (24:1) and centrifuged to sediment the particulate debris. The upper aqueous phase was transferred to a clean tube containing an equal volume of 6 M guanidinium isothiocyanate and the DNA was bound to 30 µl silica dioxide powder (Vogelstein and Gillespie, 1979). The silica-bound DNA was collected by centrifugation, resuspended twice in 75% ethanol, dried, and eluted into sterile distilled water.

2.3. PCR amplification and nucleotide sequencing

Bidirectional nucleotide sequences were determined for two nuclear intergenic regions, EFutr and Bloc. PCR amplification and sequencing primers for these loci are listed in Table 2. EFutr and Bloc were amplified by PCR: EFutr was amplified in two overlapping fragments with the primer pairs EFigs1F × R7int and F2int × EFRint; Bloc was amplified with the primer pair B5.1F and B3.1R. PCR conditions included 10–25 ng genomic DNA, 200 µM dNTPs, 0.4 µM each of two locus-specific primers (IDT, IO), 1.0 U of *Taq* DNA polymerase (Promega, Madison, WI) in 50 µl reaction volumes in 1× reaction buffer supplied by the manufacturer. Thermal cycling included an initial template denaturation at 94 °C for 2 min; 40 cycles at 94 °C for 30 s, 56 °C annealing for 30 s, 72 °C for 2 min; 72 °C extension for 15 min.

Table 2
Primer sequences for amplifying and sequencing the intergenic loci EFutr and Bloc

Locus	Primer name	Sequence (5′–3′)
EFutr forward	EFigs1F	ACCCCAAGTTCATCAAGTCTGGTGAC
	Fint	CTTCTCCTTCGCCGCGGTCTC
	F2int	ACCCGCCTCGATGCTCTCAT
	F4int	TCGCGGTGGTAGTCAATGTAC
	F5int	CTCATGGTTGCCAGGATTTAG
EFutr reverse	Rbint	AGCCAGCTGTCGACGGAAGAA
	R7int	TCCTGGCCTCGTCCGAGTTT
	EFR4	ATTTGACACAGCCCATACTCA
	EFR2	TCTGGGCCGTGCTTTAATATGG
	EFR	CAACTTCCCAAACGCCCAACC
Bloc forward	B5.1F	CGACCCGGCCAACTACTTTGA
	B22U	GTCGCAGCCAGAGCAACT
	BFint	GTTCCTTGCCCTCGGTAATGAA
Bloc reverse	BRint	AGCATATCGGGCATGACTGA
	B822L	AGATTCGCAACGTCAACTT
	B3.1R	GTCTTCCAGTACCACTACGCC

PCR products were isolated from 1.5% NuSieve agarose gels (Cambrex, Walkersville, MD) and extruded by the freeze-squeeze method (Tautz and Renz, 1983). Nucleotide sequencing was performed with BigDye Terminator Cycle Sequencing Kits on a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence data were edited with Sequencher 4.1 (GeneCodes, Ann Arbor, MI), aligned in the Megalign module of DNASTAR 5 (Lasergene, Madison, WI) and output in NEXUS file format for phylogenetic analysis.

2.4. Phylogenetic analysis

Sequence data were analyzed by maximum parsimony (MP) and Bayesian inference (BI). MP analyses were conducted with PAUP* version 4.0b10 (Swofford, 2002). Only aligned, informative nucleotide positions were included in MP analyses; all gaps and ambiguously aligned positions

were excluded. Most-parsimonious trees (MPT) were inferred by heuristic MP searches using 1000 replicates implementing the random stepwise-addition and tree bisection-reconnection (TBR) options. For phylogenetic analyses of individual loci, nucleotide characters were equally weighted and unordered. For analysis of the combined EFutr and Bloc data, nucleotide characters for each locus was weighted in inverse proportion to the data partition with the least number of informative characters. Node support was evaluated by 1000 replications of non-parametric bootstrap resampling (Felsenstein, 1985). MrBayes 3.0 b4 (Huelsenbeck, 2000; Huelsenbeck et al., 2001) was used to infer phylogenetic trees using BI (Mau et al., 1999; Rannala and Yang, 1996). BI analyses consisted of four replicate searches, each of which included four chains and 2×10^6 Markov chain Monte Carlo generations and a random tree was saved every 100 generations. Stability of likelihood scores was determined by plotting likelihood score against generation number. After stability in likelihood scores was achieved, the first 5000 "burn-in" trees were discarded. The remaining "post burn-in" trees were pooled and a 50% majority-rule determined with PAUP*.

To determine whether data from EFutr and Bloc could be combined and analyzed in a single analysis, topological incongruence between EFutr and Bloc phylogenies was assessed using a reciprocal 70% bootstrap (BP) and a 95% posterior probability (PP) criterion (Mason-Gamer and Kellogg, 1996; Reeb et al., 2004). Topological conflict was deemed significant if discordant relationships (i.e., monophyly vs. non-monophyly) received BP $\geqslant$ 70% and PP $\geqslant$ 95% for the majority-rule consensus trees of both data partitions. Different data partitions are deemed combinable if no significant conflict is detected.

Three rounds of phylogenetic analyses were conducted. An initial combined analysis of EFutr and Bloc was carried out to infer the phylogenetic origins of CBB pathogens. This included MP and BI analyses of 34 ingroup isolates, which included 26 *B. bassiana s.l.* isolates, representing a broad sampling or the geographic and phylogenetic diversity within this species complex and eight CBB pathogens from the Neotropics (6) and Africa (2). Sequences from *B. brongniartii* Petch, ARSEF 1041, was used as outgroup to root the analysis.

The second analysis was conducted to examine phylogenetic relationships within AFNEO_1, a clade resolved in the first analysis. AFNEO_1 included the majority of both African and Neotropical isolates associated with CBB, additional Neotropical isolates infecting insect species other than CBB, and three Neotropical isolates associated with coffee plants. This analysis consisted of MP and MP bootstrap searches of EFutr and Bloc sequences. Sequences from *B. bassiana* ARSEF 656 were used to root the second analysis.

A third analysis was conducted to compare the topologies of EFutr and Bloc among Neotropical AFNEO_1 isolates. Tree searches consisted of separate MP and MP bootstrap analyses for both the Bloc and EFutr data sets. The outgroup designated for this analysis was *B. bassiana* s.l. ARSEF 656. To infer historical patterns of reproductive mode, a Partition Homogeneity Test (PHT; Farris et al., 1995; Huelsenbeck et al., 1996; Geiser et al., 1998) was conducted with the isolates included in Analysis 3. This test computes the sum of the tree lengths of the MPT's for each locus. Thereafter, a series of replicate data sets are created by pooling all characters and randomly allocating them to two partitions that are equal in size to the original data. The sum of the MPT lengths from each pair of replicate partitions is calculated and these values are used to form a distribution of tree-lengths. The probability that the sum of lengths of the original MPT's lies within this distribution is calculated, with a low probability ($P < 0.05$) implying incongruence (i.e., recombination).

3. Results

3.1. Results of phylogenetic analyses

The concatenated EFutr and Bloc sequence alignment comprised 4352 nucleotide positions (2781 for EFutr and 1571 for Bloc), including gapped positions inserted to compensate for length differences among sequences due to indels. All gaps and ambiguously aligned positions were excluded from phylogenetic analyses. In the first analysis, EFutr and Bloc yielded 234 and 184 parsimony informative nucleotide positions, respectively. MP searches for each data set were conducted individually (data not shown). The MP search results for the EFutr data yielded six most parsimonious trees (MPT), length (L) = 344 steps, consistency index (CI) = 0.7674, retention index (RI) = 0.9268, rescaled consistency index (RC) = 0.7113. MP search results for Bloc were 1 MPT, L = 518, CI = 0.4170, RI = 0.6992, RC = 0.2916 (data not shown). No significant conflict was detected among the MPT's of the single locus analyses (data not shown) and the data sets were combined. In the MP analysis of the combined data the EFutr and Bloc characters were weighted 1× and 1.27×, respectively. Results of the combined EFutr and Bloc MP and BI analyses are summarized in Fig. 1. In Fig. 1 terminal clades are labeled according to their geographic origin (e.g., North America 1,2,3).

The second analysis focused on an expanded set of isolates in clade AFNEO_1 and included 46 in-group taxa, rooted to ARSEF 656. All AFNEO_1 isolates added to the second analyses were from the Neotropics and included 28 isolates from CBB, 12 isolates from insects other than CBB and three isolates associated with coffee either as epiphytes (1) or fruit endophytes (2). An additional 45 isolates isolated from CBB in Western Africa had EFutr and Bloc alleles that matched the alleles present in the African isolates previously analyzed and, therefore, were not included (data not shown). The EFutr and Bloc phylogenies and search results for Analysis 2 are shown in Fig. 2.

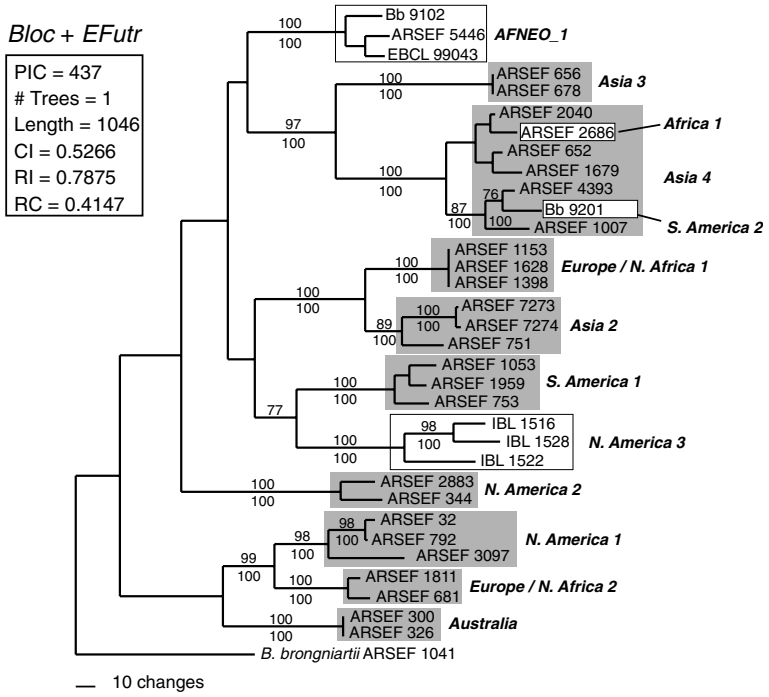

Fig. 1. The single most parsimonious tree from MP search of the combined EFutr and Bloc data sets. Definitions for the abbreviations of character and tree statistics given in the box inset are: PIC, number of parsimony informative characters; L, length of tree; CI, consistency index; RI, retention index; and RC, rescaled consistency index. MP bootstrap indices $\geqslant 70\%$ are reported above branches and posterior probabilities $\geqslant 95\%$ from Bayesian analysis are given below branches the posterior probabilities. Isolates from CBB are boxed.

A third MP analysis was conducted to contrast the topologies of the EFutr and Bloc phylogenies inferred for a subset of 29 Neotropical AFNEO_1 isolates that possessed unique combinations of EFutr and Bloc alleles. Single MPT's and accompanying tree statistics for each locus are presented in Fig. 3 and the positions of isolates in the corresponding trees is indicated by interconnecting lines. The PHT statistic was significant ($P < 0.001$); the summed tree length of the actual data was 324 steps, which was 58 steps shorter than the shortest tree produced from the synthetic data sets and 68 steps shorter than >95% of all trees.

4. Discussion

The principal objective of this study was to infer the phylogenetic origins of *B. bassiana s.l.* pathogens of CBB in Africa and the Neotropics. Specifically, we wished to determine whether the ability by *B. bassiana s.l.* to infect CBB had single or multiple origins, whether CBB-associated *B. bassiana* s.l. have tracked the intercontinental movement of coffee and CBB, or if CBB has acquired *B. bassiana s.l.* pathogens endemic to regions where this pest insect has been introduced. To this end, we have initiated development of a sequence database for nuclear intergenic regions EFutr and Bloc, which we have determined to contain sequence variation informative to phylogenetic relationships within the *B. bassiana s.l.* global complex. The purpose of this sequence database is to provide insight into the evolutionary history, biogeography, ecology and epidemiology of *B. bassiana* s.l., and to facilitate communication about these ubiquitous and ecologically prominent entomopathogens.

Based on an initial global sampling of *B. bassiana* s.l. presented here, the EFutr and Bloc phylogenies together yielded evidence for eleven significantly supported terminal clades (Analysis 1; Fig. 1). This result complements and extends recent evidence that *B. bassiana s.l.* is a complex of cryptic lineages (Rehner, 2005; Rehner and Buckley, 2005). In the present analysis, the majority of terminal clades detected are endemic to single continents and sister lineages usually have non-overlapping distributions. This biogeographic structuring of lineages suggests that isolation by distance (allopatry) has played an important role in the phylogenetic diversification of *B. bassiana s.l.*, as is the case for the majority of organisms (Mayr, 1942; Coyne and Orr, 2004). Inference of the intraspecific phylogeny of *B. bassiana s.l.* represents an important step toward clarifying speciation patterns and mechanisms within this species complex.

The recently proposed genealogical concordance phylogenetic species recognition criterion (GCPSR; Taylor et al., 2000; Matute et al., 2006) stipulates the phylogenetic concordance of gene genealogies as a basis for the diagnosis and recognition of species. Under this criterion, many of the terminal clades resolved in the present analysis qualify for recognition as species. The singular status of these terminal lineages is supported by their demonstrated ability to remain genetically distinct in regions where two or more lineages occur in sympatry (e.g., Africa, Asia, Europe, and the Americas). The evident lack of hybridization among

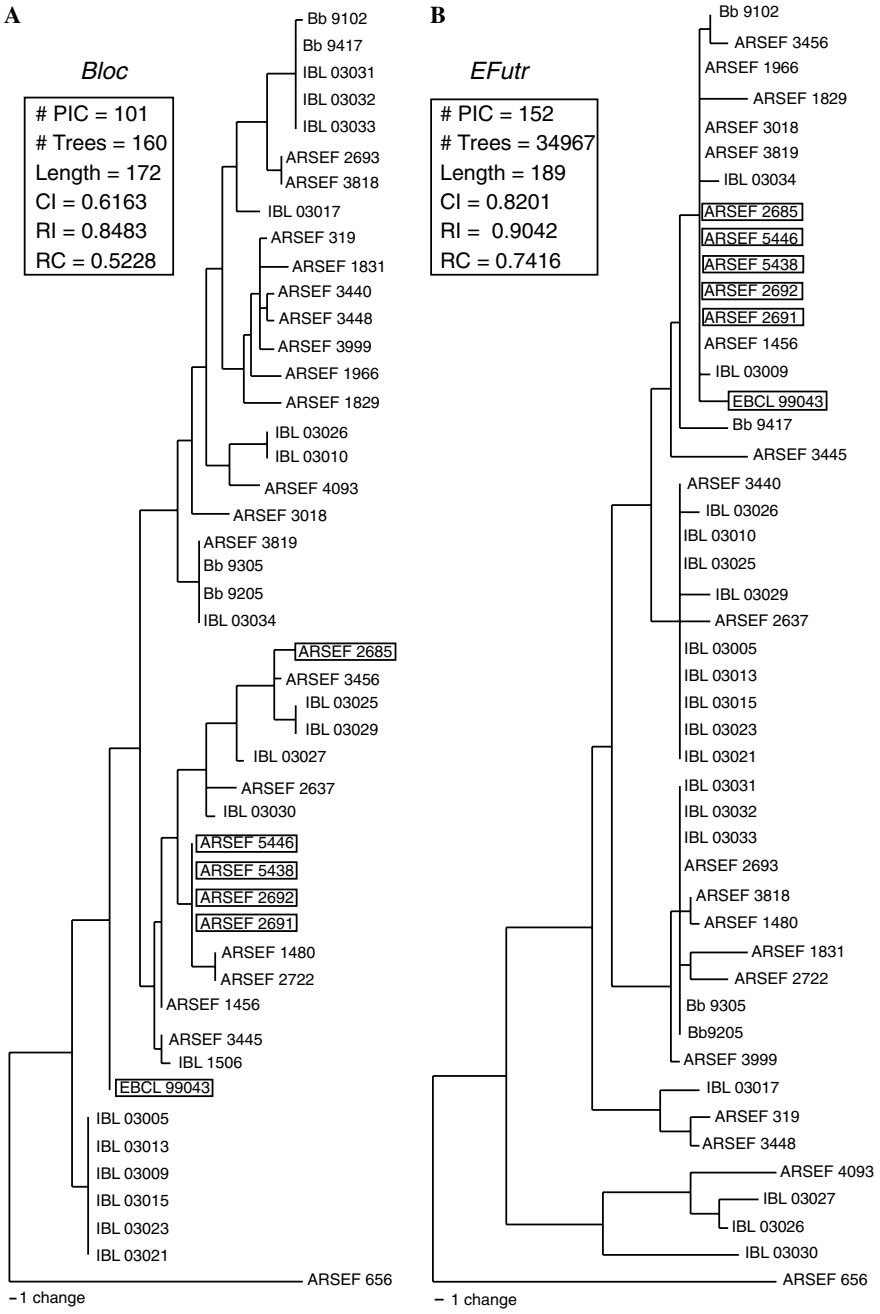

Fig. 2. Single most parsimonious trees of Bloc (A) and EFutr (B) for AFNEO_1 isolates. Tree statistics are included in box inset (abbreviations as defined in Fig. 1 legend) and isolates are indicated by their accession numbers, geographic origin and host information. Accession numbers of African isolates are enclosed in boxes.

sympatric lineages thus suggests that these phylogenetic species may also correspond to biological species. Castrillo et al. (2004) reported that genetic exchange among auxotrophic mutants was observed only among very closely related strains of *B. bassiana*, indicating the possible existence of reinforcement mechanisms that prevent hybridization among isolates from genetically divergent lineages (Kohn, 2005). Although the precedent for describing species of fungi on the basis of molecular phylogenetic criteria has been broached (e.g., Fisher et al., 2002; O'Donnell et al., 2004), implementation of the GCPSR in fungal taxonomy has not yet been widely adopted. However, phylogenetic evidence is increasingly incorporated in species revisions in cases where there are morphological or biological character differences among terminal sister lineages (e.g., Andersen et al., 2003). To date, no unique morphological, cultural, or host range characteristics have been identified that support the diagnoses of terminal lineages resolved by molecular phylogenies in *B. bassiana s.l.* (Rehner and Buckley, 2005; Rehner, unpub.). The lack of defining phenotypic characteristics that differentiate members of different lineages, the present incomplete state of knowledge of lineage diversity

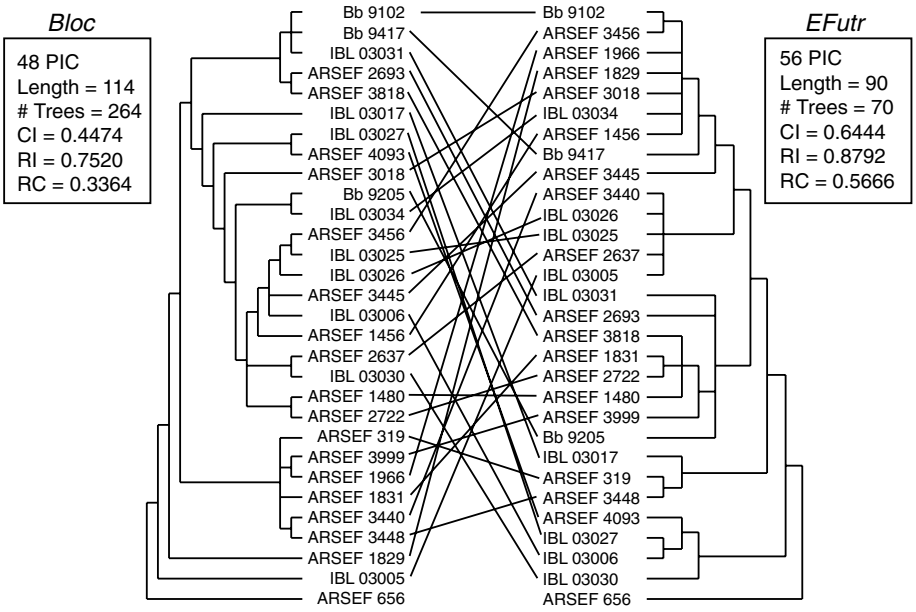

Fig. 3. Single most parsimonious trees of EFutr and Bloc for a subset of twenty-nine Neotropical AFNEO_1 isolates, each with a unique combination of EFutr and Bloc alleles. Tree statistics are included in box inset (abbreviations as defined in Fig. 1 legend) and isolates are labeled according to their accession number. Diagonal lines between cladograms indicate the position of isolates in the two gene trees to facilitate visualization of topological incongruence.

within *B. bassiana s.l.*, and the unresolved taxonomic status of *B. bassiana s.l.* argue against formally recognizing these cryptic lineages as distinct species at this time. However, molecular phylogenies, as presented here, can stand in as a stable, ad hoc, framework for identifying and communicating about these ecologically important fungi until a taxonomic revision of this species complex is available.

The combined EFutr and Bloc phylogeny revealed that isolates from four lineages of *B. bassiana s.l.* are pathogens of CBB, and these are referred to as AFNEO_1, Africa 1, S. America 2, and N. America 3 (Fig. 1). The monophyletic AFNEO_1 is a well-supported lineage of uncertain affinity and includes the majority of African and Neotropical CBB-associated isolates examined in this study (Table 1) and will be discussed in further detail below. Africa 1 and S. America 2 are both single African (Kenya) and South American (Colombia) isolates, respectively, and each is derived from within the Asia 4 clade. Additional isolates from Africa 1 and S. America 2 are needed to determine whether these non-Asian lineages constitute distinct clades within Asia 4, or whether Asia 4 is a broadly distributed group. The fourth group, N. America 2, includes three Mexican isolates, and is sister to South America 1, a clade of Brazilian isolates. These findings demonstrate that in both the Neotropics and Africa, CBB is vulnerable to infection by multiple lineages of *B. bassiana s.l.* This suggests that a potentially complex network of epidemiological and coevolutionary relationships likely exist between CBB (and other insects) and their *B. bassiana s.l.* pathogens.

Insight into the genetic structure and historical relationship between the disjunct African and Neotropical AFNEO_1 subgroups (Fig. 2) was obtained from allele genealogies of Bloc and EFutr for an expanded set of isolates (Analysis 2; Fig. 2). Total allele diversity between the Neotropical and African subgroups differed by approximately 10-fold. Among 57 African isolates, only 3 and 2 alleles were detected whereas among the 48 Neotropical isolates there were 26 and 25 alleles at Bloc and EFutr, respectively. Although African and Neotropical alleles are phylogenetically intermixed, only a single allele at EFutr was shared between the two groups (Fig. 2). The predominately mutually exclusive distribution of alleles suggests that these disjunct groups have been isolated for sufficient time during which shared ancestral alleles have largely been eliminated. On this basis, it appears that the split leading to the contemporary African and Neotropical AFNEO_1 groups probably occurred prior to the human-facilitated movement of coffee agriculture.

The wide disparity in allele diversity between the Neotropical and African AFNEO_1 groups may reflect differences in their evolutionary histories, demographic and current population structures. The high genetic diversity detected in the geographically and ecologically ubiquitous Neotropical AFNEO_1 suggests that this lineage is both endemic and well established in the Neotropics. Its emergence as the most frequently isolated fungal pathogen of the recently introduced CBB is consistent with its observed broad host range among Neotropical insects and the apparent ubiquitous occurrence of this lineage throughout the Neotropics. In contrast, the low allele diversity within Africa AFNEO_1 may signify that this group has been subject to a recent genetic bottleneck. Whether this postulated bottleneck occurred at the founding of this population in

western Africa or more recently within a subpopulation of genotypes that have shifted onto CBB (a scenario possibly enhanced by clonal amplification of these genotypes) cannot be determined with the present data. Further sampling of isolates from surrounding habitats and from other insect hosts, in conjunction with data from more sensitive genetic markers, is needed to clarify the demographic and population genetic structure of these fungi.

In Neotropical AFNEO_1 Bloc and EFutr alleles are geographically widespread and no obvious phylogeographic patterning was evident (data not shown). Visual inspection of trees from Analysis 3 (Fig. 3) revealed extensive incongruence between the Bloc and EFutr genealogies. Further, a Partition Homogeneity Test identified significant conflict in the reconstructed Bloc and EFutr phylogenies, indicating that this lineage may be historically recombining. A similar result was obtained for a cryptic species of North American *Metarhizium anisopliae* (Bidochka et al., 2005). Thus, despite the observed prevalence of clonal reproduction in *Beauveria* and *Metarhizium*, both taxa appear to be intrinsically recombining. However, much remains to be clarified as to the mode and frequency of recombination within the many lineages that constitute these cryptically diverse fungal taxa.

Although the majority of Neotropical *B. bassiana s.l.* included in this study were isolated as pathogens of CBB or other insects, three isolates were directly associated with coffee plants, either on the phylloplane (IBL 03033) or as endophytes of coffee berries (IBL 03031, 03032). Endophytism by *B. bassiana s.l.* has been observed in temperate and tropical ecosystems, most notably from maize (Lewis and Bing, 1991), potato, cotton and jimsonweed (Jones, 1994), tomato (Leckie, 2002), *Theobroma gileri* (Evans et al., 2003), as well as from a variety of forage plants (Arnold and Lewis, 2005). In laboratory inoculations, Posada et al. (2003) demonstrated that *B. bassiana s.l.* can exist endophytically in coffee. The close phylogenetic association of plant-associated *B. bassiana s.l.* isolates with CBB pathogens suggests that *B. bassiana s.l.* has considerable flexibility with respect to its positioning in the environment, ecological niche, and nutritional ecology. Whether this flexibility can be exploited in protecting coffee from its insect pests is the subject of on-going research.

A robust hypothesis for the phylogeny of *B. bassiana s.l.* permits investigation of the phylogeny, life history, ecology and biogeography of this important clade of fungal entomopathogens. Our phylogenetic analysis, based on two nuclear intergenic regions, Bloc and EFutr, indicate that the global *B. bassiana* s.l. complex is cryptically diverse and consists of multiple phylogenetic species. Using this phylogenetic framework, we identified four distinct lineages within *B. bassiana s.l.* that infect CBB in Africa and the Neotropics, although one clade, AFNEO_1, is the dominant CBB pathogen on both continents. Allelic variation at both loci enabled further insight into the genetic structure and differences between the continentally disjunct populations of AFNEO_1. Additionally, phylogenetic discordance between gene genealogies in Neotropical AFNEO_1 suggests a history of recombination in this group. Together these findings are consistent with other evidence that *B. bassiana s.l.* are primarily generalist, opportunistic, arthropod pathogens (Bidochka et al., 2002; Wang et al., 2005; Rehner and Buckley, 2005). However, the predominance of AFNEO_1 as a natural fungal entomopathogen of CBB in both Africa and the Neotropics suggests that this lineage warrants further scrutiny as a potential source of biocontrol agents against this serious pest of coffee.

Acknowledgments

We thank Richard Humber and Karen Hansen of ARSEF for providing fungal isolates. We are indebted to Joseph Bischoff and Marilena Aquino de Muro for comments on drafts of this manuscript, Faridah Dahlan for editorial comments, and anonymous reviewers for their comments. The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

References

Andersen, B., Nielsen, K.F., Thrane, U., Szaro, T., Taylor, J.W., Jarvis, B.B., 2003. Molecular and phenotypic descriptions of *Stachybotrys chlorohalonata* sp. nov. and two chemotypes of *Stachybotrys chartarum* found in water-damaged buildings. Mycologia 95, 1227–1238.

Aquino de Muro, M., Mehta, S., Moore, D., 2003. The use of amplified fragment length polymorphism for molecular analysis of *Beauveria bassiana* isolates from Kenya and other countries, and their correlation with host and geographical origin. FEMS Microbiol. Lett. 229, 249–257.

Aquino de Muro, M., Elliott, S., Moore, D., Parker, B.L., Skinner, M., Reid, W., El Bouhssini, M., 2005. Molecular characterization of *Beauveria bassiana* isolates obtained from overwintering sites of Sunn Pests (*Eurygaster* and *Aelia* species). Mycol. Res. 109, 294–306.

Arnold, A.E., Lewis, L.C., 2005. Ecology and evolution of fungal endophytes and their roles against Insects. In: Vega, F.E., Blackwell, M. (Eds.), Insect–Fungal Associations Oxford University Press, New York, pp. 74–96.

Averna-Saccá, R., 1930. Os entomophagos cryptogamicos na broca do cafeeiro, encontrados em S. Paulo. Bolm. Agric. 31 (1-24), 195–213.

Balakrishnan, M.M., Sreedharan, K., Krishnamoorthy Bhat, P., 1994. Occurrence of the entomopathogenic fungus *Beauveria bassiana* on certain coffee pests in India. J. Coffee Res. 24, 33–35.

Balakrishnan, M.M., Viyajan, V.A., Sreedharan, K., Krishnamoorthy Bhat, P., 1995. New fungal associates of the coffee berry borer *Hypothenemus hampei*. J. Coffee Res. 25, 52–54.

Barrera, J.F., Moore, D., Abraham, Y.J., Murphy, S.T., Prior, C., 1990. Biological control of the coffee berry borer, *Hypothenemus hampei*, in Mexico and possibilities of further action. Brighton Crop Prot. Conf. Pests Dis., 391–396.

Barrios, A.M., 1992. Producción y virulencia de algunas cepas del hongo entomopatógeno *Beauveria bassiana* (Bals.) Vuill. contra la broca del cafeto *Hypothenemus hampei* (Ferrari). M. S. thesis, Instituto Interamericano para Cooperación en la Agricultura—Centro Agronómico Investigación y Enseñanza.

Bautista, L., 2000. Hongos entomopatógenos parasitando en estado natural broca del café *Hypothenemus hampei* (Ferrari) (Coleoptera:

Scolytidae) en el estado Táchira, Venezuela. VI Jornadas Científico-Técnicas. Universidad Nacional Experimental del Táchira, Venezuela.
Beille, L., 1925. Les stephanoderes sur les caféiers cultivés à la Côte d'Ivoire. Revue de Botanique Appliquée et d'Agriculture Tropicale 5, 387–388.
Benavides, P., Vega, F.E., Romero-Severson, J., Bustillo, A.E., Stuart, J., 2005. Biodiversity and biogeography of an important inbred pest of coffee, the coffee berry borer, *Hypothenemus hampei* (Ferrari). Ann. Entomol. Soc. Am. 98, 359–366.
Bidochka, M.J, Menzies, F.V., Kamp, A.M., 2002. Genetic groups of the insect-pathogenic fungus *Beauveria bassiana* are associated with habitat and thermal growth preferences. Arch. Microbiol. 178, 531–537.
Bidochka, M.J., Small, C.N., Spironello, M., 2005. Recombination within sympatric cryptic species of the insect pathogenic fungus *Metarhizium anisopliae*. Env. Microbiol. 7, 1361–1368.
Bridge, P.D., Abraham, Y.J., Cornish, M.C., Prior, C., Moore, D., 1990. The chemotaxonomy of *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) isolates from the coffee berry borer *Hypothenemus hampei* (Coleoptera: Scolytidae). Mycopathologia 111, 85–90.
Brun, L.O., Marcillaud, C., Gaudichon, V., Suckling, D.M., 1989. Endosulfan resistance in *Hypothenemus hampei* (Coleoptera: Scolytidae) in New Caledonia. J. Econ. Entomol. 82, 1311–1316.
Bustillo P., A.E., Cárdenas M., R., Villalba G., D. A., Benavides M., P., Orozco H., J. Posada F. J., 1998. Manejo integrado de la broca del café *Hypothenemus hampei* (Ferrari) en Colombia. Centro Nacional de Investigaciones de Café (Cenicafé) Chinchiná, Colombia, p. 134.
Castrillo, L.A., Griggs, M.H., Vandenberg, J.D., 2004. Vegetative compatibility groups in indigenous and mass-released strains of the entomopathogenic fungus *Beauveria bassiana*: likelihood of recombination in the field. J. Invertebr. Pathol. 86, 26–37.
Coates, B.S., Hellmich, R.L., Lewis, L.C., 2002. *Beauveria bassiana* haplotype determination based on nuclear rDNA internal transcribed spacer PCR-RFLP. Mycol. Res. 106, 40–50.
Coyne, J.A., Orr, H.A., 2004. Speciation. Sinauer Associates, Sunderland, MA.
Damon, A., 2000. A review of the biology and control of the coffee berry borer, *Hypothenemus hampei* (Coleoptera: Scolytidae). Bull. Entomol. Res. 90, 453–465.
de Oliveria Filho, M.L., 1927. Contribuição para o conhecimento da broca do café *Stephanoderes hampei* (Ferrari 1867). Secretaria da Agricultura, Comercio e Obras Públicas. São Paulo, Brazil. pp. 95.
Evans, H.C., Holmes, K.A., Thomas, S.E., 2003. Endophytes and mycoparasites associated with an indigenous forest tree, *Theobroma gileri*, in Ecuador and a preliminary assessment of their potential as biocontrol agents of cocoa diseases. Mycol. Prog. 2, 149–160.
Farris, J.S., Kallersjo, M., Kluge, A.G., Bult, C., 1995. Testing significance of incongruence. Cladistics 10, 152–158.
Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.
Fernãndez, P.M., Lecuona, R.E., Alves, S., 1985. Patogenicidade de *Beauveria bassiana* (Bals) Bullí. a broca do cafe *Hypothenemus hampei* (Ferrari 1867) Coleoptera. Scolytidae. Ecossistema 10, 176–182.
Fisher, M.C., Koenig, G.L., White, T.J., Taylor, J.W., 2002. Molecular and phenotypic description of *Coccidioides posadasii* sp. nov., previously recognized as the non-Califonia population of *Coccidioides immitis*. Mycologia 94, 73–84.
Friederichs, K., Bally, W., 1923. Over de parasitische schimmels, die den Koffiebessenboeboek dooden. Meded. Koffiebessenboeboek-fonds 6, 103–147.
ffrench-Constant, R.H., Steichen, J.C., Brun, L.O., 1994. A molecular diagnostic for endosulfan insecticide resistance in the coffee berry borer *Hypothenemus hampei* (Coleoptera: Scolytidae). Bull. Entomol. Res. 84, 11–16.
Gaitan, A., Valderrama, A.M., Saldarriaga, G., Vélez, P., Bustillo, A., 2002. Genetic variability of *Beauveria bassiana* associated with the coffee berry borer *Hypothenemus hampei* and other insects. Mycol. Res. 106, 1307–1314.
Geiser, D., Pitt, J.I., Taylor, J.W., 1998. Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. Proc. Natl. Acad. Sci. USA 95, 388–393.
Glare, T.R., Inwood, A.J., 1998. Morphological and genetic characterization of *Beauveria* spp. from New Zealand. Mycol. Res. 102, 250–256.
Goettel, M.S., Inglis, G.D., 1997. Fungi: Hyphomycetes. In: Manual of Techniques in Insect Pathology Academic Press, San Diego, CA, pp. 213–249.
Hagedorn, M., 1910. Wieder ein neuer kaffeeschädlinge. Entomologische Blätter 6, 1–14.
Huelsenbeck, J.P., 2000. MrBayes: Bayesian Inferences of Phylogeny (Software). University of Rochester, New York.
Huelsenbeck, J.P., Bull, J.J., Cunningham, C.W., 1996. Combining data in phylogenetic analysis. Trends Ecol. Evol. 11, 152–158.
Huelsenbeck, J.P., Nielsen, E.S., Bollback, J.P., 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294, 2310–2314.
Jones, K.D., 1994. Aspects of the Biology and Biological Control of the European Corn Borer in North Carolina. PhD Dissertation, North Carolina State University.
Klein-Koch, C., Espinoza, O., Tandazo, A., Cisneros, P., Delgado, D., 1988. Factores naturales de regulación y control biologico de la broca del café (*Hypothenemus hampei* Ferr). Sanidad Vegetal (Ecuador) 3, 5–30.
Kohn, L.M., 2005. Mechanisms of fungal speciation. Annu. Rev. Phytopathol. 43, 279–308.
Lazo, A.R,R., 1990. Susceptibilidad de la broca del fruto del cafeto *Hypothenemus hampei* al hongo entomopatógeno *Beauveria bassiana*, y su tolerancia al oxicloruro de cobre. M. S. thesis, Instituto Interamericano para Cooperación en la Agricultura—Centro Agronómico Tropical de Investigación y Enseñanza, Turrialba, Costa Rica.
Leckie, B.M., 2002. Effects of *Beauveria bassiana* mycelia and metabolites incorporated into synthetic diet and fed to larval *Helicoverpa zea*, and detection of endophytic *Beauveria bassiana* in tomato plant using PCR and ITS. M.S. thesis. The University of Tennessee, Knoxville, p. 75.
Le Pelley, R.H., 1968. Pests of Coffee. Longmans, Green & Co., Ltd, London. p. 590.
Lewis, L.C., Bing, L.A., 1991. *Bacillus thuringiensis* Berliner and *Beauveria bassiana* (Balsomo) Vuillemin for European corn borer control: program for immediate and season-long suppression. Can. Entomol. 123, 387–393.
Mason-Gamer, R.J., Kellogg, E.A., 1996. Testing for phylogenetic conflict among molecular data sets in the Triticeae (Gramineae). Syst. Biol. 45, 524–545.
Matute, D.R., McEwen, J.G., Montes, B.A., San-Blas, G., Bagagli, E.l., Rauscher, J.T., Restrepo, A., Morais, F., NinoVega, G., Taylor, J.W., 2006. Cryptic speciation and recombination in the fungus *Paracoccidioides brasiliensis* as revealed by gene genealogies. Mol. Biol. Evol. 23, 65–73.
Mau, B., Newton, M., Larget, B., 1999. Bayesian phylogenetic inference via Markov chain Monte Carlo methods. Biometrics 55, 1–12.
Maurer, P., Couteaudier, P.A., Girard, P.D., Bridge, P.D., Riba, G., 1997. Genetic diversity of *B. bassiana* and relatedness to host insect range. Mycol. Res. 101, 159–164.
Mayr, E., 1942. Systematics and the Origin of Species. Columbia University Press, New York.
Méndez-López, I., 1990. Control microbiano de la broca del fruto del cafeto *Hypothenemus hampei* Ferrari (Coleoptera Scolytidae), con el hongo *Beauveria bassiana* (Bals.) Vuill. (Deuteromycetes) en el Soconusco, Chiapas. M. S. thesis, Colegio de Postgraduados, Chapingo, Mexico, p. 135.
Monterroso, J.L., 1981. Incidencia del *Beauveria bassiana* sobre la broca del café y su reproducción en coco en Guatemala. Revista Cafetalera (Guatemala) 210, 10–12.
Moore, D., Prior, C., 1988. Present status of biological control of the coffee berry borer *Hypothenemus hampei*. Brighton Crop Prot. Conf. Pests Dis. vol. 3, pp. 1119–1124.
Moore, D., Prior, C., Abraham, Y. J., 1990. Current developments and future potential in biological control of the coffee berry borer *Hypothenemus hampei* Ferrari with emphasis on the use of *Beauveria bassiana*. In: Proceedings of the International DLG-Symposium on Integrated Pest Management in Tropical and Subtropical Systems, Bad Dürkheim, Federal Republic of Germany, February 8–15, 1989, pp. 313–321.

Murphy, S.T., Moore, D., 1990. Biological control of the coffee berry borer *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae): previous programmes and possibilities for the future. Biocon. News Infor. 11, 107–117.

O'Donnell, K., Ward, T.J., Geiser, D.M., Kistler, H.C., Aoki, T., 2004. Concordance between the mating type locus and seven other nuclear genes support formal recognition of nine phylogenetically distinct species with the *Fusarium graminearum* clade. Fungal Genet. Biol. 41, 600–623.

Pascalet, P., 1939. La lutte biologique contre *Stephanoderes hampei* ou scolyte du caféier au Cameroon. Revue de Botanique Apliquée & d'Agriculture Tropicale Bulletin 219, 753–764.

Pérez, J., Infante, F., Vega, F.E., Holguín, F., Macías, J., Valle, J., Nieto, G., Peterson, S.W., Kurtzman, C.P., O'Donnell, K., 2003. Mycobiota associated with the coffee berry borer *Hypothenemus hampei* (Coleoptera, Scolytidae) in Chiapas, Mexico. Mycol. Res. 107, 879–887.

Posada, F.J., Vega, F.E., Rehner, S.A., 2003. *Beauveria* as a possible coffee endophyte. Annual Meeting of the Society for Invertebrate Pathology, Burlington, VT, July 26–31, p. 47.

Posada-Flórez, F.J., Marín-Marín, P., Pérez-Sierra, M., 1998. *Paecilomyces lilacinus*, enemigo natural de adultos de *Hypothenemus hampei*. Cenicafé 49, 72–77.

Rannala, A., Yang, Z.H., 1996. Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. J. Mol. Evol. 43, 304–311.

Reeb, V., Lutzoni, F., Roux, C., 2004. Contribution of RPB2 to multilocus phylogenetic studies of the Euascomycetes (Pezizomycotina, Fungi) with special emphasis on lichen-forming Acarosporaceae and evolution of polyspory. J. Mol. Phyl. Evol. 32, 1036–1060.

Rehner, S.A, 2005. Phylogenetics of the insect pathogenic genus *Beauveria*. In: Vega, F.E., Blackwell, M. (Eds.), Insect-Fungal Associations: Ecology and Evolution Oxford University Press, New York, NY, pp. 3–27.

Rehner, S.A., Buckley, E.P., 2005. A *Beauveria* phylogeny inferred from nuclear ITS and EF1-α sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. Mycologia 97, 84–98.

Steyaert, R.L., 1936. Un ennemi natural du *Stephanoderes*: le *Beauveria bassiana*. Étude Agronom. Congo Belge No. 2, October 1936, p. 46.

Swofford D.L., 2002. PAUP*: Phylogenetic Analysis using Parsimony (and other methods) Version 4.0b10. Sinauer Associates, Inc., Sunderland, Mass.

Tautz, D., Renz, M., 1983. An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels. Anal. Biochem. 132, 14–19.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.S., Fisher, M.C., 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genet. Biol. 31, 21–32.

Vega, F.E., Mercadier, G., 1998. Insects, coffee, and ochratoxin A. Fla Entomol. 81, 543–544.

Vega, F.E., Mercadier, G., Damon, A., Kirk, A., 1999. Natural enemies of the coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae) in Togo and Ivory Coast, and additional entomofauna associated with coffee beans. Afr. Entomol. 7, 243–248.

Vega, F.E., Mercadier, G. Dowd, P. F., 2000. Fungi associated with the coffee berry borer *Hypothenemus hampei* Ferrari (Coleoptera: Scolytidae). In: Proceedings of the 18th International Scientific Colloquium on Coffee, Helsinki, August 1999. Association Scientifique Internationale du Café (ASIC), pp. 229–238.

Vélez-Arango, P., Benavides-Gómez, M., 1990. Registro e identificación de *Beauveria bassiana* en Ancuya, departamento de Nariño, Colombia. Cenicafé (Colombia) 41, 50–57.

Vogelstein, B., Gillespie, D., 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA 76, 615–619.

Wang, C., Shah, F.A., Patel, N., Li, Z., Butt, T.M., 2005. Molecular investigation on strain relatedness and population structure of *Beauveria bassiana*. Env. Microbiol. 5, 908–915.

Waterhouse, D.F., Norris, K.R., 1989. Biological Control: Pacific Prospects—Supplement 1. ACIAR Monograph No. 12, p. 125.