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Full Length Research

Molecular characterization and the effectiveness of native entomopathogenic *Beauveria bassiana* strains against adults of Mediterranean fruit fly (*Ceratitis capitata*)

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ABSTRACT: Molecular characterization of locally isolated *Beauveria bassiana* (*Cordycipitaceae*, *Hypocreales*) strains and their pathogenicity against *Ceratitis capitata* (*Diptera: Tephritidae*) adults are herein reported for the first time. Molecular identification based on the phylogenetic analyses of ITS-rDNA and combined data of *TEF1* and Bloc, revealed that all fungal strains were conclusively assigned to *B. bassiana* clade. Interestingly, Maximum parsimony based-phylogeny analysis of the combined *TEF1* and Bloc sequences detected additional genotypes among the sub-clades than ITS-rDNA analysis. Genetic diversity among strains was performed using random amplified polymorphic DNA (RAPD) markers. RAPD genotyping segregated all the strains into three clusters and characterized the 15 strains with different genotypes. RAPD-PCR clustering analysis showed that *B. bassiana* strains were relatively separated according to geographical origin. Molecular analyses using nuclear genes (*TEF1* and Bloc) and RAPD markers suggest that *B. bassiana* is an aggregate of species, rather than a single species. Under laboratory conditions, *B. bassiana* strains were highly pathogenic to *C. capitata* adults with mortality rate in the range of 71.67–91.67%. Based on the Average of Survival Time (AST) and median lethal times (LT_{50}) values, fungal strains were able to reduce significantly lifespan of flies compared to controls. Further experiments under field conditions are required to evaluate the real contribution of these fungal strains as promising biological control agents against *C. capitata* adults in Morocco.

Keywords: Biocontrol, Bloc, *ETF1,* Morocco, phylogenetic analyses, RAPD-PCR.

INTRODUCTION

Mediterranean fruit fly (Medfly) *Ceratitis capitata* (Wiedermann) (*Diptera: Tephritidae*) is the most economically important species of fruit flies among *Tephritidae* throughout the world (White and Elson-Harris, 1994). In Morocco, Medfly takes from *Argania spinosa* forests the main refuge, from which continuously invades the surrounded agricultural crops, inducing heavy damages particularly to citrus orchards (Alaoui et al., 2010; Imoulan et al., 2011). As the fruit fly larvae feed and develop on the host pulp, fruit become rotten and inedible causing large direct economic losses (D'Aquino et al., 2011). So far, adult control measures have typically relied on the use of broad-spectrum chemical insecticides (Imoulan and El Meziane, 2013). However, the overuse of conventional insecticides has resulted in serious ecological imbalance (Arouri et al., 2015), raising the concern about the sustainability of the traditional approach for pest control.

As alternatives to chemical control, several microorganisms can serve as a promising approach to

control fruit flies (Ekesi et al., 2007; Quesada-Moraga et al., 2006). Among these microbial agents, appears *Beauveria bassiana* (*Cordycipitaceae*, *Hypocreales*), an entomopathogenic fungus well-known by its potency to kill and to regulate insect population (Butt and Goettel, 2000). Unlike other microbial pathogens, entomopathogenic fungi have the advantage to infect and colonize their host through the host cuticle (Goettel et al., 2005). This property has prompted extensive researches in order to develop the efficient strains as biological control agents (Inglis et al., 2001). In Morocco, biological control approach remains at the embryonic scale with few works being published on the insect pathogenic fungi as biocontrol agents alongside to those established worldwide (Imoulan et al., 2011; Imoulan and El Meziane, 2013). These studies showed that some *B. bassiana* strains displayed high levels of pathogenicity against puparia and third instar larvae of Medfly, producing a large mortality rate ranging from 65 to 95% and caused significant reduction in the emergence of adult flies.

Beauveria is easily distinguished at genus level using morphological features, while species identification is complicated with the extensive overlap in morphological characteristics among species. Recently, all the species within this genus were well resolved using nuclear genes adapting the phylogenetic species recognition concept (Rehner et al., 2011). Genetic variability among strains have been performed using different molecular markers, providing more evidence on the diversity among strains than through morphological features solely. Molecular characterization using DNA-based methods such as RAPD-PCR analysis (Bidochka et al., 1994) can help, indeed, to distinguish individual strains in order to develop a specific marker for the environmental monitoring of the strains after being released in areas infested by *C. capitata*.

In Morocco, no detailed studies have been conducted on molecular characterization of *Beauveria* and their effectiveness against adult of *C. capitata*. In this study, we aimed to investigate the biological and molecular characterization of a subsample of *Beauveria* strains recovered from Moroccan forests of *A. spinosa*. The identification at species level was carried out using sequencing data from three nuclear genes and genetic diversity was assessed using RAPD-PCR genotyping. The antagonistic potential of the fungal strains was also evaluated under laboratory conditions against Medfly using adult stage. This investigation will help to raise the likelihood of a successful outcome in the biological control effort against Medfly in this country.

MATERIALS AND METHODS

Fungal strains and growth conditions

Beauveria strains were recovered from soil samples collected from *A. spinosa* forests in Morocco using *Galleria mellonella* baiting method (Imoulan et al., 2011). A total of 15 most pathogenic strains were selected from previous large screening for promising candidates using *C. capitata* pupae (Imoulan et al., 2011). To establish monosporic cultures, conidial suspensions of 1×10^5 conidia ml^{-1} were prepared and plated on PDA plates. The single colony propagated from single conidia was transferred into a new PDA dish and incubated at 25 °C.

Genomic DNA extraction, PCR and sequencing

Strains of *Beauveria* recovered from single conidia were grown as mycelia for 2 weeks in 250-ml flasks containing 100 ml of potato dextrose broth. Cultures were then shaken at 150 rpm on a rotary shaker at 25 °C for 7 days in darkness. The mycelial samples were pelleted from liquid cultures by centrifugation for 10 min at 3500 rpm (Eppendorf AG, Hamburg, Germany), subsequently washed twice with sterile double distilled water, and then lyophilized and stored at −80 °C.

About 100 mg of lyophilized mycelia for each strain was grounded in a sterile mortar using a pestle. Total genomic DNA was extracted following the modified CTAB method described by Yao et al. (1999). The mixtures were incubated for 2 hours at 65 °C, then extracted twice with 24 : 1 chloroform : isoamyl alcohol, and centrifuged at 10,000 rpm at 4 °C for 15 min. The supernatant was transferred to a clean tube and mixed with 1/10 volume of sodium acetate (3 M, pH 5.2). Total genomic DNA was precipitated by adding 2/3 (v/v) of cold isopropanol and chilled overnight at −20 °C. DNA was recovered by centrifugation at 10,000 rpm, 4 °C for 10 min and washed twice with 70% alcohol, dried at room temperature and resuspended in TE buffer (10 Mm Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0). The extracted DNA was stored at −20 °C until use.

Genomic DNA was used as template for PCR amplification of ITS regions (White et al., 1990), Elongation Factor 1-alpha (*TEF1*) (Rehner et al., 2005) and the Bloc nuclear intergenic (Rehner et al., 2006) using the following primer pairs ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3')/ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), 983F (5'-GCYCCYGGHCAYCGTGAYTTYAT-3')/ 2218R (5'-ATGACACC RACRGCRACRGTYTG-3') and B5.1F (5'-CGACCCGGCCAACTACTTTGA-3')/ B3.1R (5'-GTCTTCCAGTACCACTACGCC-3'), respectively. The PCR reactions were performed in a final volume of 50 µl containing 25 µl 2 × Taq PCR Master Mix (Tiangen Biotech Co., LTD, China), 0.5 µl of each primer (10 µM), 10 ng of genomic DNA and 23 µl of RNase-Free water, and run in a thermocycler (Veriti®, Applied Biosystems, USA). Cycling conditions for amplification of ITS regions

consisted of 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 53 °C, 1 min at 72 °C and final extension step of 10 min at 72 °C. Concerning the amplification of *TEF1* gene, cycling conditions was performed using a touchdown procedure with 2 min initial denaturation at 94 °C followed by 10 cycles of 30 s at 94 °C, 30 s at 66 °C which was subsequently incrementally reduced by 1 °C per cycle, and 1 min at 72°C. An additional 36 amplification cycles were then performed with the same conditions, however, with a fixed annealing temperature of 56 °C and a final extension of 10 min at 72 °C. The cycling conditions of Bloc consisted of 3 min initial denaturation at 94°C for 3 min followed by 35 cycles of 30 s at 94 °C, 30 s at 56 °C, 1 min at 72 °C and a final extension of 10 min at 72 °C. PCR products were checked by electrophoresis on an agarose gel (1%) at 100 V in 0.5 × TAE buffer (40 mM Tris-Acetic acid, pH 8.0, 1mM EDTA), and sequenced with corresponding primers described above. The sequencing was performed by Beijing Genomics Institute (Beijing, China) by using a capillary sequencer (Applied Biosystems 3730 Analyzer, Foster City, California). All the sequences used in this study were submitted to GenBank with accession numbers given in Table 1.

Phylogenetic and data analyses

All DNA sequences were assembled and edited manually as necessary using BioEdit ver. 7.2.5 (Hall, 1999). A total of 29 representative sequences of recognized species of *Beauveria* and one sequence of *Cordyceps militaris* (ARSEF 5050), the out group, were retrieved from GenBank (Table 1) and included in this study. Multiple sequence alignments were made using the Clustal W program (Thompson et al., 1997). Data sets of *TEF1* and Bloc were concatenated into a single file using SequenceMatrix (Vaidya et al., 2011) and analyzed with Maximum parsimony (MP). ITS sequences were excluded from the phylogenetic analysis given of its limited information within ingroups (Rehner et al., 2011).

MP analysis was computed with PAUP* (Swofford, 2003) using 1000 replicates of heuristic search option of random sequence additions with branch swapping algorithm by tree bisection reconnection (TBR) under equal character weighting and MulTrees on. Branches of zero length were collapsed, and all parsimonious trees were saved. Alignment gaps were treated as missing data. Branch support was estimated by bootstrap analysis with 1,000 replicates (Felsenstein, 1985) executing the same search strategy as described above.

Molecular characterization

The RAPD-PCR reactions were carried out in a total volume of 25 µL containing 2.5 µl of Taq polymerase buffer (10 ×), 0.2 mM dNTP, 0.01 mM primer, 2 mM $MgCl_2$, 1U *Taq* DNA polymerase (Promega) and 10 ng of DNA. The thermocycler program included an initial denaturation at 94 °C for 2 min followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 35 °C for 1 min, and extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min. RAPD products were separated by electrophoresis on 1.2% agarose gels. The molecular sizes of the amplified DNA bands were estimated using 1 kb ladder (Promega). Initially, 21 RAPD primers (OPC and OPT kits from Operon) were screened, out of which, only five most polymorph reproducible RAPD primers with easily recordable bands were selected for genetic diversity analysis.

To score RAPD markers, only clear and well resolved bands were analyzed as RAPD loci. Data from the RAPD banding patterns were coded in a binary data matrix of presence (1) or absence (0). Genetic similarity among the genotypes was estimated using Dice index of similarity, and the dendrogram was constructed using the UPMGA clustering method (Sneath and Sokal, 1973). All computations were performed using the NTSYSpc 2.1 package (Numerical Taxonomy System, Applied Biostatistics, Setauket, New York) (Rohlf, 2000).

Pathogenicity against Medfly adults

Insects

Field-infested fruits with Medfly larvae were collected from *A. spinosa* forests and kept in polyethylene containers with sterilized sand at 25 ± 2 °C and a relative humidity of 65%. Third instar larvae and pupae were collected daily by sifting the sand. Upon emergence, the flies were translocated in cages sized 40 × 40 × 40 cm and provided with mixture of sugar and yeast (3:1) and water. Insects were kept under laboratory conditions for several generations and the tenth one was used for the following pathogenicity tests.

Inoculum, viability and bioassays

Conidia were harvested by scraping the surface of 3-week-old cultures and suspended in 10 ml of sterile aqueous solution of 0.01% Tween 80. Conidial concentrations were determined using a haemocytometer following serial dilution in sterile distilled water. A standard initial concentration of 1×10^8 conidia ml^{-1} was made for each strain. The viability of conidia was determined by spread-plating 0.1 ml of conidial suspension titrated to 3×10^6 conidia ml^{-1} on PDA plates. Sterile microscope cover slips were placed on each plate and incubated at 25 °C. Percentage germination was scored after 20 h at 200× magnification by counting 100 conidia randomly for each replicate. Conidia were

Table 1 Strains used in *Beauveria* phylogenetic analysis, their origin, substrate and GenBank numbers

Strain	Species	Locality	Host/Substrate	GenBank number		
				ITS	Bloc	*TEF*
OUJ4.111	*B. bassiana*	Morocco, North	Soil	**KT378221**	**KU663905**	**KT748529**
OUJ6.124	*B. bassiana*	Morocco, North	Soil	**KT378222**	**KU663906**	**KT748530**
OUJ18.118	*B. bassiana*	Morocco, North	Soil	**KT378226**	**KU663907**	**KT748531**
OUJ18.120	*B. bassiana*	Morocco, North	Soil	**KT378227**	**KU663908**	**KT748532**
OUJ18.122	*B. bassiana*	Morocco, North	Soil	**KT378228**	**KU663909**	**KT748533**
OUJ5.32	*B. bassiana*	Morocco, North	Soil	**KT378230**	**KU663910**	**KT748534**
OUJ20.75	*B. bassiana*	Morocco, North	Soil	**KT378231**	**KU663911**	**KT748535**
OUJ14.96	*B. bassiana*	Morocco, North	Soil	**KT378225**	**KU663912**	**KT748536**
ESR1.1	*B. bassiana*	Morocco, West	Soil	**KT378218**	**KU663913**	**KT748537**
ESR4.16	*B. bassiana*	Morocco, West	Soil	**KT378219**	**KU663914**	**KT748538**
SMI2.31	*B. bassiana*	Morocco, West	Soil	**KT378220**	**KU663915**	**KT748539**
TAM6.2	*B. bassiana*	Morocco, West	Soil	**KT378229**	**KU663916**	**KT748540**
AML1.289	*B. bassiana*	Morocco, South	Soil	**KT378223**	**KU663917**	**KT748541**
AMS8.366	*B. bassiana*	Morocco, South	Soil	**KT378224**	**KU663918**	**KT748542**
BAH4.94	*B. bassiana*	Morocco, South	Soil	**KT378232**	**KU663919**	**KT748543**
ARSEF 300	*B. bassiana*	Australia	*Hemiptera: Lygaeidae*	AY532015	HQ880690	AY531924
ARSEF 751	*B. bassiana*	Vietnam	*Coleoptera: Chrysomelidae*	AY532045	HQ880694	AY531954
ARSEF 1040	*B. bassiana*	Japan	*Lepidoptera: Bombycidae*	AY531972	HQ880689	AY531881
ARSEF 4622	*B. australis*	Australia	*Orthoptera: Acridiidae*	HQ880788	HQ880721	HQ880996
ARSEF 4598	*B. australis*	Australia	Soil	HQ880789	HQ880720	HQ880995
ARSEF 617	*B. brongniartii*	France	*Coleoptera: Scarabaeidae*	HQ880782	HQ880713	HQ880991
ARSEF 10278	*B. brongniartii*	USA, Oregon	Soil: Rhizosphere	HQ880769	HQ880700	HQ880979
ARSEF 10280	*B. brongniartii*	USA, Oregon	Soil: Rhizosphere	HQ880771	HQ880702	HQ880981
ARSEF 4474	*B. asiatica*	China	*Coleoptera: Scarabaeidae*	AY531936	HQ880717	AY531936
ARSEF 4850	*B. asiatica*	Republic of Korea	*Coleoptera: Cerambycidae*	AY531936	HQ880718	AY531937
ARSEF 7279	*B. sungii*	Republic of Korea	*Coleoptera: Scarabaeidae*	HQ880813	HQ880744	HQ881009
ARSEF 7280	*B. sungii*	Republic of Korea	*Coleoptera: Scarabaeidae*	HQ880814	HQ880745	HQ881010
ARSEF 7281	*B. sungii*	Republic of Korea	*Coleoptera: Scarabaeidae*	HQ880815	HQ880746	HQ881011
ARSEF 7760	*B. malawiensis*	Malawi	*Coleoptera: Cerambycidae*	DQ376247	HQ880756	DQ376246
ARSEF 4755	*B. malawiensis*	Australia	Soil	HQ880825	HQ880754	HQ881015
ARSEF 7117	*B. caledonica*	USA, Georgia	*Orthoptera: Gryllacrididae*	HQ880820	HQ880751	HQ881013
ARSEF 8024	*B. caledonica*	Denmark	*Coleoptera: Scarabaeidae*	HQ880818	HQ880749	HQ881012

*GenBank accession numbers in bold are sequences generated in this study.
NA: not available.

Table 1. Contd.

ARSEF 4149	*B. amorpha*	Australia	*Coleoptera: Scarabaeidae*	HQ880804	HQ880735	HQ881006
ARSEF 7542	*B. amorpha*	USA, Colorado	*Hymenoptera: Formicidae*	HQ880805	HQ880736	HQ881007
ARSEF 1855	*B. pseudobassiana*	Canada	*Coleoptera: Scolytidae*	HQ880796	HQ880727	HQ880999
ARSEF 2997	*B. pseudobassiana*	Canada	*Hymenoptera: Vesidae*	HQ880797	HQ880728	HQ881000
ARSEF 3216	*B. pseudobassiana*	USA, Wisconsin	*Thysanoptera: Thripidae*	AY532019	HQ880725	AY531927
ARSEF 2694	*B. varroae*	Switzerland	*Coleoptera: Curculionidae*	HQ880802	HQ880733	HQ881004
ARSEF 8257	*B. varroae*	France	*Acari: Varroidae*	HQ880800	HQ880731	HQ881002
ARSEF 8259	*B. varroae*	France	*Acari: Varroidae*	HQ880801	HQ880732	HQ881003
RCEF 3903	*B. sinensis*	China, Anhui	*Lepidoptera: Geometridae*	HM135176	NA	HQ270151
RCEF 5500	*B. lii*	China,Shaanxi	*Coleoptera: Coccinellidae*	JN689372	JN689373	JN689371
ARSEF 7032	*B. kipukae*	USA, Hawaii	*Homoptera: Delphacidae*	HQ880803	HQ880734	HQ881005
AESEF 2922	*B. vermiconia*	Chile	Soil	AY532012	HQ880753	AY531920
ARSEF 5050	*Cordyceps militaris*	USA, New York	*Lepidoptera*	HQ880829	NA	HQ881020

considered to have germinated when the germ tube was longer than the conidia diameter.

Laboratory bioassays were undertaken in controlled environment room at 25 ± 2 °C, 65% RH and 12:12 (L:D) photoperiod. Newly molted adults (20–24 h) that had previously been cold anesthetized were treated with 2 µl of the conidial suspension of 10^8 conidia ml^{-1} on ventral surface of the abdomen. Controls were treated with the same volume of a sterile aqueous solution of 0.01% Tween 80. The treated flies were then released into cages (40 × 40 × 40 cm) and provided with water and mixture of sugar and yeast (3:1). Each treatment was replicated three times with 40 insects per replicate and all the bioassays were tested at the same time. Mortality was recorded daily and dead flies were removed to prevent horizontal transmission of conidia. Dead insects were immediately surface sterilized with 1% sodium hypochlorite for 1 min, followed by three rinses with sterile distilled water. The cadavers were subsequently transferred to Petri dishes lined with damp sterilized filter paper and kept at 25 °C to be inspected for fungal external growth. Mortality due to fungal infection was confirmed by microscopic examination.

Statistical analysis

Mortality data were normalized through Arcsine transformation and then examined using one-way analysis of variance (ANOVA) followed by mean separation by the Student-Newman-Keuls (SNK) test ($P = 0.05$). Time to kill 50% of the insects (LT_{50}) values was estimated using the probit analysis method for correlated dada (Throne et al., 1995). The average of survival time (AST) of adults was performed using the Kaplan-Meier survival analysis.

RESULTS

Molecular identification and phylogenetic analysis

PCR amplification of rDNA-ITS region consistently gave a single strong product of about 560 bp in all examined *Beauveria* strains. Sequencing of the PCR amplicons, followed by Blast analysis, indicated that ITS sequences had 99 to 100% homology to those named *B. bassiana* in GenBank. MP analysis of ITS sequences including 29 representative sequences of recognized species of *Beauveria* clustered all Moroccan strains into *B. bassiana* clade, farther subdivided to three major sub-clades (data not shown).

PCR amplicons generated by sequencing for *TEF1* and Bloc yielded a molecular size of approximately 1100 and 1500 bp for all the strains, respectively. The combined alignment of *TEF1* and Bloc included a total of 2553 characters alignment comprising a total of 45 sequences, including 15 local *Beauveria* strains and 29 recognized *Beauveria* strains as well as *Cordyceps militaris* (ARSEF 5050) as outgroup. After the ambiguously aligned positions excluded, the final alignment comprised a total of 2553 characters (*TEF1*: 992; Bloc: 1561), of which 1800 were constant, 209 parsimony-uninformative and 544 parsimony-

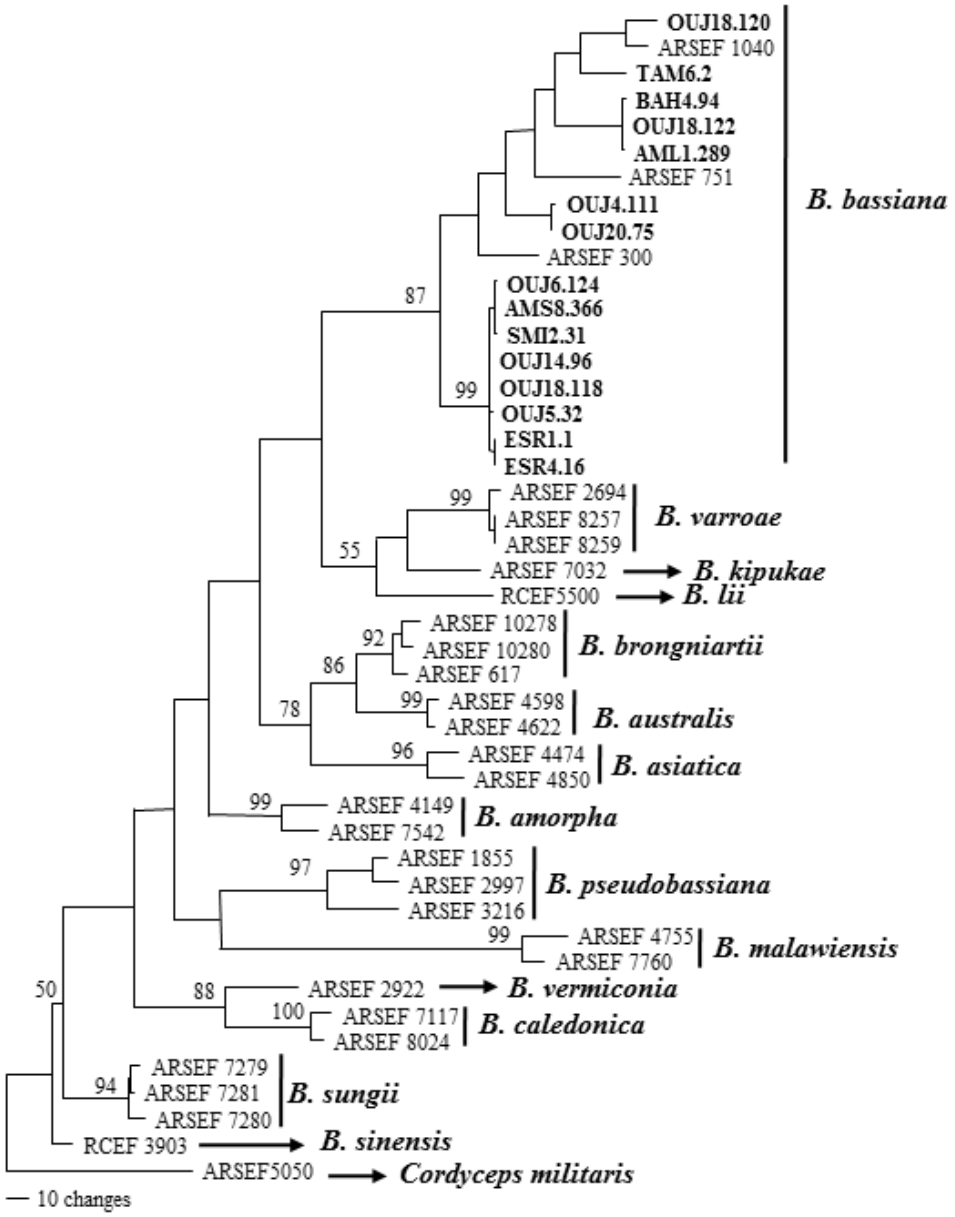

Figure 1. Phylogenetic tree of *Beauveria* based on Maximum parsimony of combined data of *TFE1* and Bloc sequences. Bootstrap values (≥50%) are labeled above branches. Terminal clades are labeled according to ARSEF accession numbers of individual isolates reported in Rehner et al. (2011), except RCEF5500 and RCEF3903 (Zang et al., 2012; Chan et al., 2013). Moroccan *B. bassiana* strains are in bold.

informative characters. The heuristic search yielded 61 most-parsimonious trees with tree length (L) 1462, consistency index (CI) 0.617, homoplasy index HI (0.383) and retention index (RI) 0.828.

MP phylogenetic analysis of the combined sequence dataset consistently resolved all *Beauveria* lineages in separated terminal branches with high bootstrap support values (Figure 1). This phylogenetic analysis clustered the 15 Moroccan *Beauveria* strains into *B. bassiana* clade with a bootstrap support of 87%, which additionally partitioned on four distinct subclades (Figure 1). This result was consistent with the MP phylogenetic relationship based on ITS sequences (data not shown). However, phylogenetic analysis based combined data was more reliable in detecting additional genotypes within *B. bassiana* sub-clades (Figure 1).

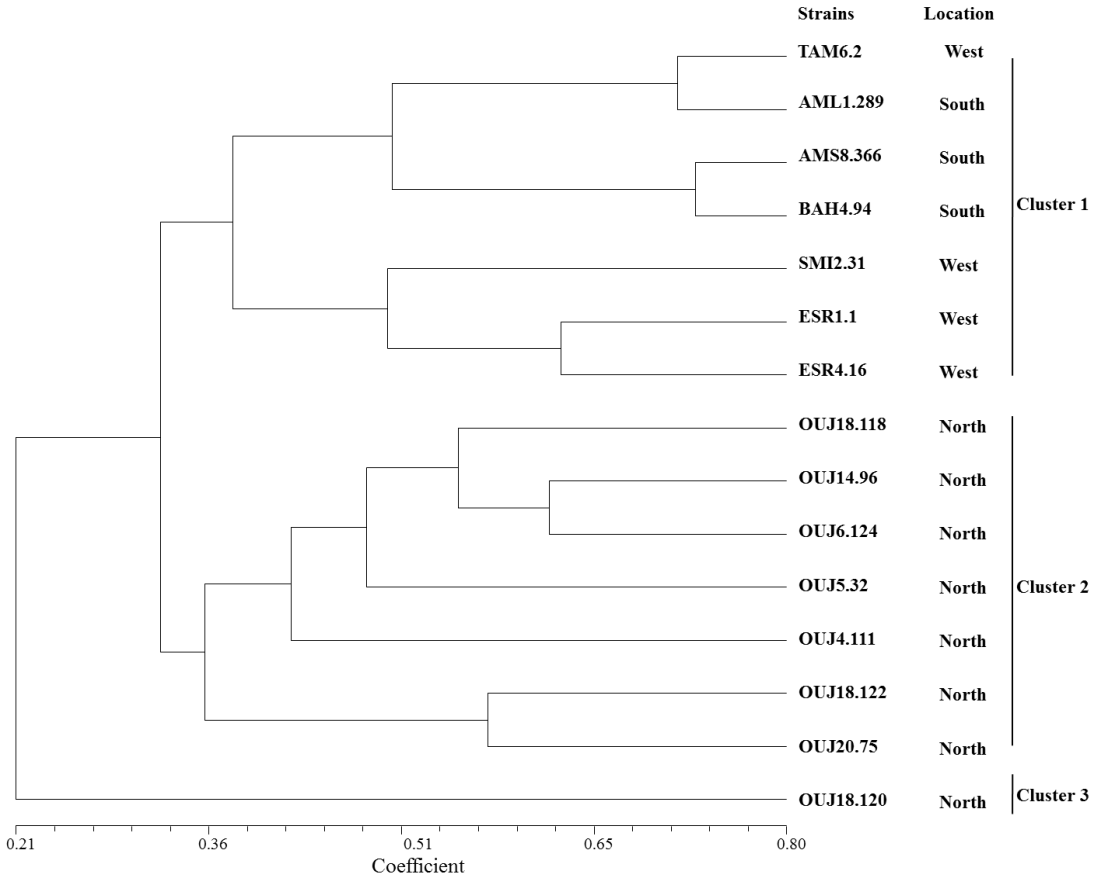

Figure 2. Dendrogram of 15 *B. bassiana* strains constructed using genetic similarity analysis based on molecular profiles revealed by five RAPD markers.

Molecular characterization

The five RAPD-PCR markers have generated reproducible and multiple polymorphic DNA fragments. The RAPD patterns revealed a total of 128 well-resolved and consistent bands out of which 98 bands being polymorphic. The primers amplified between 10 (OPC5) and 34 (OPT13) RAPD bands with an average of 25.6. The size of the amplification products ranged from 180 (OPT16) to 3790 bp (OPT14). Cluster analysis of RAPD data showed that all strains were characterized by different genotypes, indicating high genetic diversity among the strains (Figure 2). As shown in UPGMA phylogenetic tree (Figure. 2), RAPD patterns yielded three major clusters. The two major clusters each consisted of seven strains and the third group has only one strain totally diverse as compared to remaining *B. bassiana* strains. Strains from west and south of Morocco were grouped in the first cluster which further distinguished into two sub-clades, respect almost to their origin of isolation. *B. bassiana* isolated from the northern location were grouped together in the second cluster. The genetic similarity among the strains was in the range of 0.13 and 0.73. *B. bassiana* strains BAH4.94 and AMS8.366 shared the highest genetic similarity and the lowest variability was recorded between *B. bassiana* strains OUJ14.96 and OUJ18.120.

Pathogenicity against Medfly adults

In viability tests, germination of conidia ranged from 85 to 96% after 20 h. Mortality in controls was 5.84% at the end of bioassays and no mycoses with *B. bassiana* were recorded. Medfly was highly susceptible to all the fungal strains after being treated with conidial suspensions. There was a significant effect of *B. bassiana* on adult mortality compared with untreated *C. capitata* adults, with mean values between 71.67 and91.67% (Table 2). There was no significant difference in the pathogenicity among strains (F = 1.004, df = 14, P >0.5). The most virulent strains to *C. capitata* adults were OUJ18.118 and

Table 2. Susceptibility of *C. capitata* adults to fifteen locally isolated *B. bassiana* under laboratory conditions.

Strain	Pathogenicity to *C. capitata* adults				
	Mortality	Regression analysis of time mortality		Kaplan-Meier survival analysis	
	(means ± SE)*, %	LT_{50}, d	95% C.I	AST(means ± SE)**, d	95% C.I
ESR1.1	85.00 ± 5.00^{ab}	6.97	6.42-7.48	7.40 ± 0.50^{abc}	6.42-8.38
ESR4.16	86.67 ± 6.01^{ab}	5.44	4.92-5.92	6.32 ± 0.40^{a}	5.54-7.10
SMI2.31	80.00 ± 5.00^{ab}	7.22	6.74-7.72	7.73 ± 0.41^{bc}	6.93-8.54
OUJ4.111	85.00 ± 2.89^{ab}	8.84	8.33-9.40	9.73 ± 0.49^{e}	8.76-10.70
OUJ6.124	78.33 ± 6.67^{ab}	8.40	7.42-9.81	8.48 ± 0.19^{c}	8.11-8.86
AML1.289	76.67 ± 1.67^{ab}	9.63	8.75-10.63	8.21 ±0 .33^{bc}	7.57-8.86
AMS8.366	86.67 ± 4.41^{ab}	9.06	8.46-9.67	9.39 ± 0.41^{e}	8.58-10.19
OUJ14.96	85.00 ± 2.89^{ab}	8.04	7.61-8.48	8.98 ± 0.38^{abc}	6.86-8.36
OUJ18.118	91.67 ± 3.33^{a}	6.92	6.49-7.35	7.61 ± 0.47^{c}	8.06-9.89
OUJ18.120	85.00 ± 2.89^{ab}	6.6	5.83-7.31	6.90 ± 0.47^{abc}	5.97-7.83
OUJ18.122	90.00 ± 5.78^{a}	5.64	5.13-6.1	6.63 ± 0.44^{ab}	5.77-7.49
TAM6.2	81.67 ± 7.27^{ab}	7.81	7.47-9.17	7.90 ± 0.25^{bc}	7.42-8.39
OUJ5.32	86.67 ± 7.27^{ab}	8.19	7.47-9.17	8.09 ± 0.32^{bc}	7.46-8.72
OUJ20.75	76.67 ± 8.82^{ab}	10.23	9.59-10.93	9.72 ± 0.25^{e}	9.22-10.22
BAH4.94	71.67 ± 6.67^{c}	10.28	9.89-10.71	9.62 ± 0.26^{e}	9.10-10.13

*Means within a column followed by the same letter are not significantly different. **Data in the same column followed by the same letter are not significantly different. **SE:** Standard Error; **C.I:** Confidence interval; **d:** day.

OUJ18.122 causing mortality rates of 91.67 and 90%, respectively (Table 2).

The lethal time to 50% mortality (LT_{50}) values were significantly different among the fungal isolates (F = 6.9, df = 14, P < 0.0001), varying from 5.44 to 10.28 d (Table 2). The most pathogenic strains had LT_{50} values < 6 d, and all the strains were able to kill flies faster compared with controls (Table 2). The average survival times (AST) of flies varied from 6.32 to 9.73 days. AST of Medfly adults was reduced significantly after being treated with *B. bassiana* (log-rank (Mantel-Cox), p < 0.0001) compared with untreated *C. capitata* adults (Table 2).

Discussion

In a previous study, few Moroccan *Beauveria* strains were identified as *B. bassiana* based on ITS sequences homology using Blast Network service (Imoulan et al., 2011). In this study, additional strains were identified at species level using phylogenetic analyses of three nuclear loci (ITS, *TEF1* and Bloc). Genetic diversity among the strains was performed using five RAPD markers and their effectiveness was assessed against *C. capitata* adults under laboratory conditions. MP phylogenetic analyses inferred from ITS-rDNA and combined data of *TEF1* and Bloc, showed that all *Beauveria* strains were conclusively assigned to *B. bassiana*. Interestingly, MP phylogenetic analysis of the combined dataset was more informative by detecting additional genotypes within strains indicating that this lineage harbors cryptic diversity which conformed to the study of Rehner and Buckley (2005).

RAPD markers are suitable for genetic variability analyses because they are randomly generated from many loci throughout the genome. Different level of genetic diversity of *Beauveria* sp. was reported in previous studies, involving different molecular techniques (Jie and Liangen, 2010; Poeaim et al., 2014; Garrido-Jurado et al., 2015). The five tested RAPD-PCR markers provided high discrimination among strains and characterizing all the individuals by typical RAPD profiles, suggesting alongside with phylogenetic analysis of combined dataset that *B. bassiana* is an aggregate of species rather one species (Rehner and Buckley, 2005). These distinctive RAPD profiles would be useful tool for ecological studies and for long-term monitoring of the strains dispersal, efficacy and persistence after being released into the environment (Urtz and Rice, 1997; Castrillo et al., 2003; Carneiro et al., 2008).

RAPD analyses detected different genotypes strains obtained from the same geographic sites, indicating that the genetic diversity level is not related with isolation distance of fungal strains (Fegan et al., 1993). However, previous evidence reported that isolation by distance could increases genetic diversification of *B. bassiana* (Rehner et al., 2006; Fernandes et al., 2009). According to the clustering analysis of the RAPD patterns, genetic

diversity was correlated with the geographic origin of strains and, however, no link with virulence was observed, consistent with the studies of Kaur and Padmaja (2008) and Poeaim et al. (2014). To validate this hypothesis, additional fungal strains from different geographic origin and diverse habitats are needed for further analyses.

This study reports for the first time the susceptibility of Medfly adults to Moroccan *B. bassiana* strains. The bioassays against *C. capitata* adults were performed to achieve previous assessments carried out using puparia and larvae instars of *C. capitata* (Imoulan et al., 2011; Imoulan and El Meziane, 2013) in order to develop bioinsecticides to control this pest in Morocco. The tested strains were obtained from a prior large screening for promising candidates using *C. capitata* pupae (Imoulan et al., 2011). The bioassays revealed that Moroccan *B. bassiana* had narrow intraspecific variations in mycosis levels (71.67−91.67%) which could be related to the co-evolution of *B. bassiana* with its host in *A. Spinosa* forests, where *C. capitata* is the sole representative species of *Tephritidae* (Naamani, 2004; Alaoui et al., 2010). However, large variations in the pathogenicity among *B. bassiana* strains have been reported in several previous studies (Dimbi et al., 2003; Quesada-Moraga et al., 2006).

LT_{50} values (5.44−6.97 d) are in the range of those reported by Quesada-Moraga et al. (2006) (4.6–6.1 days), and Qazzaz et al. (2015) (3.9–5.6 days). However they are appreciably shorter than those indicated by Konstantopoulou and Mazomenos (2005) (10.7–21.4 days) and Castillo et al. (2000) (5.6–11.0 days). In addition, AST values provided in Quesada-Moraga et al. (2006) were in the range of 5.4 and 14.4 days. Interestingly, some Moroccan *B. bassiana* strains caused AST values lower than 7 days. According to AST and LT_{50} values, tested fungal stains are not only virulent to Medfly adults but would help greatly to reduce the population size and lifespan of flies before the infestation of fruits by females as far as the egg laying starts 7 days after adult emergence.

The evaluation of locally isolated *B. bassiana* is potentially useful for selecting the efficient candidates as biocontrol agents to Medfly, particularly when the pathogens tend to be used locally for crop protection. This study and our previous published works suggest that the 15 tested strains could be used as promising biological control agents towards Medfly or as a part of its integrated pest management program. Given of the ineffectiveness of commercial bioinsecticides provided by Naturalis® (Intrachem Bio Italia, Italy) against several insect pests in Morocco (Dr. S. Franceschini, personal communication), native pathogens ecologically compatible with local environmental conditions and with less impact on non-target organism would be the most useful microbial agents as alternative to conventional systemic insecticides (Inglis et al., 2001).

In conclusion, 15 locally isolated *Beauveria* strains were identified as *B. bassiana* using phylogenetic analyses of three nuclear genes. The genetic variability among the strains was determined using PCR-RAPD technique and a high genetic diversity was detected. In addition, the strains had a notable potential effectiveness against Medfly adults, suggesting their usefulness as biocontrol agents against Medfly for crops protection. Different RAPD profiles will help to monitor stains dispersal and persistence after being introduced in the environment infested with *C. capitata*. Additional research is needed to determine the effectiveness of these strains under field conditions to assess certainly their real contribution as a promising biological control agent against Medfly.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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