Molecular detection and differentiation of Brazilian and African isolates of the entomopathogen *Neozygites tanajoae* (Entomophthorales: *Neozygitaceae*) with PCR using specific primers

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(Received 4 October 2008; returned 22 October 2008; accepted 29 October 2008)

Neozygites tanajoae is an entomopathogenic fungus which has been used for biocontrol of the cassava green mite (*Mononychellus tanajoa*, CGM) in Africa. Establishment and dispersal of Brazilian isolates which have been introduced into some African countries in recent years to improve CGM control was followed with specific PCR assays. Two primer pairs, NEOSSU_F/NEOSSU_R and 8DDC_F/8DDC_R, were used to differentiate isolates collected from several locations in Brazil and from three countries in Africa, Benin, Ghana and Tanzania. The first primer pair enabled the species-specific detection of *Neozygites tanajoae*, while the second differentiated the Brazilian isolates from those of other geographical origin. PCR assays were designed for detection of fungal DNA in the matrix of dead infested mites since *N. tanajoae* is difficult to isolate and culture on selective artificial media. Our results show that all isolates (Brazilian and African) that sporulated on mummified mites were amplified with the first primer pair confirming their *Neozygites tanajoae* identity. The second pair amplified DNA from all the Brazilian isolates, but did not amplify any DNA samples from the African isolates. None of the two primers showed amplification neither from any of the non-sporulating mite extracts nor from the dead uninfected mites used as negative controls. We confirmed that the two primer pairs tested are suitable for the detection and differential identification of *N. tanajoae* isolates from Brazil and Africa and that they are useful to monitor the establishment and spread of the Brazilian isolates of *N. tanajoae* introduced into Benin or into other African countries for improvement of CGM biocontrol.

Keywords: *Mononychellus tanajoa*; *Neozygites tanajoae*; molecular differentiation; microbial control; Cassava

Introduction

The fungal pathogen *Neozygites tanajoae* Delalibera Jr, Humber & Hajek (Zygomycetes: Entomophthorales) is being used in Africa as a biological control agent against the introduced cassava green mite (CGM), *Mononychellus tanajoa* (Bondar) (Acari: Tetranychidae). This fungus was initially referred to as *Neozygites*

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sp. (Delalibera, Sosa Gomez, de-Moraes, Alentar de, and Farias Araujo 1992) and later as *Neozygites floridana* (Oduor, Yaninek, van der Geest, and de-Moraes 1995, Keller 1997; Elliot et al. 2000). Since 1988, when this pathogen was first found in Brazil, considerable data have been accumulated on the epizootiological, morphological and physiological aspects of strains from Brazil, Colombia and Benin (West Africa). Knowledge about CGM-pathogenic *Neozygites* has been well documented. Consistent differences appeared with *N. floridana* (Weiser & Muma) Remaud. & Keller, which has been found to be a common pathogen of many tetranychid mites. Indeed, *N. tanajoae* is specific to CGM (Delalibera, Hajek, and Humber 2004) and has been therefore released as a biocontrol agent at various sites in West African cassava fields. Post-release monitoring in Benin has shown much higher infection rates in CGM populations in release fields compared to pre-release infection rates (Delalibera 2002; Hountondji, Lomer, Hanna, Cherry, and Dara 2002a). However, although observations from experimental release fields provided evidence for establishment and better performance of the Brazilian isolates, accurate techniques for differentiating *N. tanajoae* isolates from post-release field collections were lacking. Morphological observations of *N. tanajoae* isolates from Brazil generally revealed similarities with *N. floridana* and also with other mite pathogenic species of *Neozygites*, including African strains (Delalibera et al. 2004). Furthermore, phenotypic similarities occurred also among fungal species coexisting in the same habitat. In the search for means to separate different isolates or species of the fungus, molecular tools were thought to help in overcoming the weaknesses of the phenotypic diagnostic methods (e.g. Lee, White, and Taylor 1993; Bonants et al. 1997; Brasier, Cooke, and Duncan 1999; Judelson and Tooley 2000). Therefore, molecular techniques have been recently put in place for the differentiation of *N. tanajoae* isolates from Brazil and Africa (Delalibera et al. 2004). PCR assays based on amplification of the ITS region of the ribosomal DNA (18S rDNA) and Random Amplified Polymorphic DNA (RAPD) techniques with several primers were tested to differentiate *N. tanajoae* isolates, however, without success (Delalibera et al. 2004). Alternatively, an Amplified Fragment Length Polymorphism (AFLP) technique that integrates larger parts of the genome and that is able to detect even minor differences was developed (Delalibera 2002). As a result, oligonucleotide primers for PCR based detection of *N. tanajoae* have been designed that are capable of both differentiating this fungus from other fungal organisms and to distinguish Brazilian from African isolates. However, the development of these probes was based on Brazilian strains and the only African strain, which was collected in Benin. In the present study, we aimed at validating these probes on a larger collection of isolates from several locations in Brazil and on indigenous strains from Benin, Ghana and Tanzania collected before the introduction of Brazilian strains of *N. tanajoae* to West Africa.

Materials and methods

Fungal isolates

Of the 18 isolates (Table 1) used in this study, three were obtained recently from Brazil (collected by G.J. de Moraes in 2007), while the remaining 15 were received from the International Institute of Tropical Agriculture (IITA, Biological Control

Table 1. Viability of *Neozygites tanajoae* on various mite samples tested with the *in vivo* sporulation assay.

Label	Samples	Location of collection	Country	Year	Sporulation/Viability
B	Uninfected CGM	IITA-Benin	Benin	2007	−
C	*O. gossypii*	IITA-Benin	Benin	2007	−
D	*T. urticae*	IITA-Benin	Benin	2007	−
E	*T. aripo*	IITA-Benin	Benin	2007	−
F	Colal	Colas das almas	Brazil	1995	+
G	Altal	Alegre	Brazil	1995	+
H	Alto alegre	Alto Alegre	Brazil	2007	+
I	Cruz	Cruz	Brazil	2007	+
J	Piritiba	Piritiba	Brazil	2007	+
K	Coton	Cotonou	Benin	1995	+
L	Nampong	North Nampong	Ghana	2002	+
M	Hô	West Hô	Ghana	2002	+
N	Tanzania 1	Lusahunga	Tanzania	2005	+
O	Tanzania 3	Ngara	Tanzania	2005	+
P	Tanzania 6	kayenze	Tanzania	2005	+
Q	Tanzania 7	Kamena	Tanzania	2005	+
R	Tanzania 8	Kayenze	Tanzania	2005	+
S	Tanzania 10	Nungwe/Geita	Tanzania	2005	+

'−' indicates that the capilliconidia are absent and the sample is not infected by *Neozygites tamjoae*.
'+' indicates that sample shows capilliconidia and is infected by *Neozygites tanajoae*.

Center for Africa in Benin). Among the 15 isolates, two were supposed to be also of Brazilian origin (labelled Altal and Colal) that had been subcultured at IITA in preparation for field releases. These two isolates were imported by IITA from Alto Alegre and Cruz das Almas (both in state of Bahia in Brazil) in 1995 and kept in culture on *M. tanajoa*. The remaining 13 isolates were collected through IITA by R. Hanna from Ghana, Tanzania and Benin. Uninfected mites (*Mononychellus tanajoa*, *Oligonychus gossypii*, *Tetranychus urticae* and *Typhlodromalus aripo*) were used as negative controls for the species-specific primers. The isolates were preserved at 4°C as hyphal bodies in mummified mites on dry cotton wool inside tightly sealed vials containing glycerol in the laboratory of the Division of Plant Pathology and Plant Protection at the Georg-August University, Germany. To prevent loss of fungal viability, cultures were renewed at approximately 6-month intervals by infecting healthy mites (*in vivo* culture; see below).

Viability testing of samples by sporulation test

A laboratory test for viability was conducted at the Division of Plant Pathology and Plant Protection in Göttingen, Germany, during 2006/2007 with each of the 18 isolates used in this study. Viability was assessed with a sporulation test, in which at least five CGM mummies per fungal isolate were incubated singly on a cassava leaf disc (1.5 cm in diameter) placed on a moist cotton pad on the bottom of a plastic bottle ($190 \times 150 \times 50$ mm). Bottles were placed in a climate chamber for 24 h at 19 ± 1°C, 95 ± 5% RH and 12:12 h (light:dark) photoperiod

as used successfully for *N. tanajoae* sporulation by Oduor, de-Moraes, van der Geest, and Yaninek (1996). After 24 h of incubation, mummies were checked under the binocular microscope for sporulation (e.g. production of capilliconidia). The presence of spores released on leaf discs was an indicator of sample viability. Isolates for which at least five mummies sporulated were considered viable. Viable samples were multiplied *in vivo* (see later) and stored separately for DNA extraction.

In vivo *multiplication of* N. tanajoae

Members of the genus *Neozygites* (Gustafsson 1965; Le Rü, Silvie, and Papierok 1985; Saito, Kubota, and Shimazu 1989), and particularly *N. tanajoae* (Delalibera, Hajek, and Humber 2003), are difficult to culture on artificial media. Although *in vitro* culture of *N. floridana* is possible (Butt and Humber 1989; Leite, Smith, de-Moraes, and Roberts 2000), attempts to grow *N. tanajoae* on the same media as used for *N. floridana* from *T. urticae* were not successful (Delalibera et al. 2003). Therefore, *N. tanajoae* isolates used for DNA extraction were obtained by *in vivo* production following Dara, Lomer, and Hountondji (1998). *M. tanajoa* killed and mummified by *N. tanajoae* were put on cassava leaf discs under conditions to enable fungal sporulation as described above. At the end of this period, leaf discs were checked under a compound microscope, and those found with capilliconidia (infective *N. tanajoae* spores) spread over about two-thirds of the leaf disc surface were retained for CGM exposure. Twenty healthy adult females of *M. tanajoa* were exposed to capilliconidia on each leaf disc inside 150-mm diameter Petri dishes containing moist cotton wool and incubated at 28°C under a 12:12 h (light:dark) regime for 24 h for infection. After 4–7 days, fresh mummies were collected and stored for use in DNA extraction. These experimental conditions normally resulted in a 60–70% infection rate (Dara, Lomer, Hountondji, and Yaninek 1998; Hountondji, Yaninek, de-Moraes, and Oduor 2002b). With this technique, large numbers of freshly infected mites (mummies) were produced and immediately stored at 4°C or at −20°C for long-term storage depending on when they were to be used for DNA extraction.

Preparation of samples for DNA extraction

A single mummified mite was used for DNA extraction because of the unavailability of pure fungal material. Each mummified or non-infected mite cadaver was placed inside a PCR tube (0.5 mL) containing 10 μL of autoclaved distilled water. The PCR tubes were then covered with a thermal adhesive sealing film and left at 25°C overnight to soften the cadavers and to promote sporulation by the fungus (I. Delalibera, unpublished data). Nucleic acids of each sample were extracted using the InstaGene™ Matrix, Easy DNA kit (Bio-Rad Laboratories, Hercules, CA). DNA extraction followed the manufacturer's instructions for fungal tissues with slight modifications. The same procedure was used for DNA preparation from uninfected mites that were used as negative controls. A total of 180 samples (10 replicate samples from each of 18 isolates) was prepared for PCR analysis.

In the following step of DNA extraction, 50–100 μL of InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) were added to each PCR tube (0.5 mL) using a

1-mL pipette tip. Total DNA of each isolate was extracted from the sample prepared with InstaGene Matrix according to the manufacturer's instructions given for fungal DNA extraction. The procedure was as follows: the content of PCR tubes (0.5 mL) was incubated at 56°C for 15–30 min, vortexed at high speed for 10 s and were placed at 100°C in a boiling water bath for 8 min, then vortexed again at high speed for 10 s and centrifuged at 10,000–12,000 rpm for 2–3 min. Finally, the DNA was dissolved in 20–40 μL of the resulting supernatant, saved in a new tube and stored at −20°C until use.

PCR for detection and differentiation of Neozygites tanajoae *isolates*

The genomic DNA obtained from uninfected and infected mites was used in a PCR assay with a pair of primers, NEOSSU_F/NEOSSU_R, designed for identification of *N. tanajoae* using 18S rRNA sequences from different isolates of the fungus (I. Delalibera et al., unpublished data). The primer sequences of NEOSSU_F were 5′-GGT TTG ATT CCG GAG ATG GA-3′ and of NEOSSU_R 5′-ATA CAA CCT GCT AAG GC T GCA-3′. The amplification reactions were performed in a PTC-100™ thermocycler (MJ Research, Inc.) in 25-μL volume containing 0.2 μL each of forward and reverse primers (20 μM), 12.5 μL of Promega Master Mix (Promega PCR Master Mix, 2X: 50 units/mL of Taq DNA Polymerase supplied in a commercial reaction buffer: pH 8.5, 400 μM of each dATP, dGTP, dCTP and dTTP, 3 mM MgCl2), 10.1 μL of nuclease-free water (deionised) and 2 μL of DNA sample pipetted into 23 μL of PCR master mix. The sterile deionised water and DNA of uninfected mites were used as negative controls and in this case, 2 μL of corresponding DNA from an uninfected mite and 2 μL of sterile water (instead of template DNA) were pipetted into the 0.5-mL microcentrifuge tube containing 23 μL of PCR master mix. All reaction components, except for the primers, were purchased from MBI Fermentas (St. Leon Roth, Germany). Thermocycler reactions were programmed as follows: hot start at 94°C; initial denaturation at 94°C for 4 min; 38 cycles of denaturation at 94°C for 1 min; annealing at 50 or 52°C for 1.5 min, and extension at 72°C for 2.5 min; final extension at 72°C for 5 min and cool down to 8°C. The PCR product was stored at −20°C until use.

Differentiation of Brazilian and African isolates was done with the oligonucleotide primer pair 8DDC_F/8DDC_R. The primer sequences were based on the sequencing of a randomly amplified polymorphic DNA fragment proven to be specific for Brazilian isolates (I. Delalibera et al., unpublished data). The respective primers were based on the sequence elements 8DDC_F: 5′-TCG TGT TGG AAG CAC GTT TA-3′ and 8DDC_R: 5′-TTG ACG AAA TAG AGG CGA AAA-3′. PCR conditions and amplicon storage followed the same protocol as for the procedure described above for NEOSSU primers.

In addition, both oligonucleotide primer pairs NEOSSU_F/NEOSSU_R and 8DDC_F/8DDC_R were combined in a multiplex PCR for detection and differentiation of fungal samples. For this reaction, PCR amplification was performed under the same conditions as described above.

All PCR analyses were performed and reproduced at least six times for each DNA sample under two annealing temperatures (50 or 52°C). The photographed bands that were consistently amplified were scored. GeneRuler™100-bp ladder Plus (Fermentas, St. Leon Roth, Germany) was used as the DNA size marker.

To visualise amplicons, 10 μL of the PCR products from each isolate were loaded in a gel containing 1.5% agarose in 0.5 × TBE buffer (Tris, pH 8, boric acid, EDTA). Gels were run for 1 h at 60 V, stained with ethidium bromide, visualised with UV light and photographed.

Results

Viability of samples

A series of 180 DNA extracts was tested derived from 18 mite samples infected or uninfected with different isolates of *N. tanajoae* in order to check the specificity of the two pairs of *N. tanajoae* primers (Table 1). Samples exposed to sporulation conditions showed two types of responses. The first type consisted of dead cadaver mites with absence of capilliconidia (Table 1). These were dead uninfected mites from different species (*Mononychellus tanajoa*, *Olygonychus gossypii*, *Tetranychus urticae and Typhlodromalus aripo*) and represented the negative control for primer evaluation. The second type comprised 14 isolates with the presence of capilliconidia representing infected CGM mites which were used as positive controls in the primer evaluation.

PCR product optimisation using specific primers

The optimal annealing temperature, a critical factor for optimal PCR product formation, differed with the template-primer system (Peres, Harakava, Carroll, Adaskaveg, and Timmer 2006). PCR analysis performed for each DNA sample with two annealing temperatures (50 or 52°C) indicated that the highest yields of specific products based on NEOSSU and 8DDC primers were obtained at 52°C, where all expected bands were clearly developed. At 50°C, only faint non-specific bands were detected (image not shown).

Evaluation of species-specific primers NEOSSU

The NEOSSU primer pair was evaluated for its ability to detect DNA from *N. tanajoae* in a matrix of mite cadavers and to differentiate uninfected from infected mites (mummies). Among 180 DNA samples obtained from 18 mite samples (10 separate DNA extracts per sample origin) which included African isolates, Brazilian isolates and uninfected mites, NEOSSU primers amplified only DNA from infected mites (Table 2). PCR amplification of DNA from infected mites with (NEOSSU_F/ NEOSSU_R) resulted in reproducible bands of the expected size (800 bp) with 140 DNA samples out of 180 samples tested, thus separating the total of 14 infected mites (African and Brazilian isolates) from DNA samples from uninfected mites (Figure 1). Thus, the NESSOU primers yielded clear and specific amplicons for DNA of *N. tanajoae* at 52°C annealing temperature (Table 2, Figure 1).

Evaluation of 8DDC primers for differentiating the origin of fungal strains

The 8DDC_F/8DDC_R primer pair was evaluated for the differentiation of Brazilian and African isolates in samples that had been previously tested positive

Table 2. Isolates of *Neozygites tanajoae* amplified by the two specific pairs of primers NEOSSU_F/NEOSSU_R and 8DDC_F/8DDC_R in a polymerase chain reaction (PCR) at 52°C annealing temperature.

Label	Samples	Location of collection	Country	Viability	NEOSSU	8DDC
B	Uninfected CGM	IITA-Benin	Benin	Not infected	−	−
C	*O. gossypii*	IITA-Benin	Benin	Not infected	−	−
D	*T. urticae*	IITA-Benin	Benin	Not infected	−	−
E	*T. aripo*	IITA-Benin	Benin	Not infected	−	−
F	Colal	Colas das almas	Brazil	Infected	+	+
G	Altal	Alegre	Brazil	Infected	+	+
H	Alto alegre	Alto Alegre	Brazil	Infected	+	+
I	Cruz	Cruz	Brazil	Infected	+	+
J	Piritiba	Piritiba	Brazil	Infected	+	+
K	Coton	Cotonou	Benin	Infected	+	−
L	Nampong	North Nampong	Ghana	Infected	+	−
M	Hô	West Hô	Ghana	Infected	+	−
N	Tanzania 1	Lusahunga	Tanzania	Infected	+	−
O	Tanzania 3	Ngara	Tanzania	Infected	+	−
P	Tanzania 6	Kayenze	Tanzania	Infected	+	−
Q	Tanzania 7	Kamena	Tanzania	Infected	+	−
R	Tanzania 8	Kayenze	Tanzania	Infected	+	−
S	Tanzania 10	Nungwe/Geita	Tanzania	Infected	+	−

−indicates that the concerned primers have not amplified DNA of suspected sample. +indicates that the concerned primers amplified DNA sample.

for the presence of *N. tanajoae*. Among the 14 isolates scored as positive by the NEOSSU primer pair, DNA of five isolates was amplified with the 8DDC primer pair yielding a typical band of 600 bp (Figure 2). In fact, all the five isolates detected in this PCR were of Brazilian origin (samples Colal, Altal, Alto Alegre, Cruz and Piritiba), whereas the DNA from the nine remaining isolates did not amplify with 8DDC, as well as uninfected mites as negative control (Table 2, Figure 2).

Evaluation of primer pairs in a multiplex PCR

In the multiplex reaction with NEOSSU and 8DDC primers, all samples from isolates of *N. tanajoae* which were found viable during the sporulation test yielded the species-specific 800-bp amplicon with the NEOSSU primer pair (Figure 3). In contrast, neither sterile water nor DNA samples from uninfected mites displayed the 800-bp *N. tanajoae* diagnostic band (Figure 3). In addition, only Brazilian isolates of *N. tanajoae* produced a second band at 600 bp with the 8DDC primers in the PCR reaction (Figure 3).

Discussion

The objective of our research was to test DNA probes for identifying and differentiating *N. tanajoae* isolates collected from a broader range of locations, allowing for a large-scale analysis of the entomopathogen population in the cassava-growing regions of the world. For this, we used an international collection of

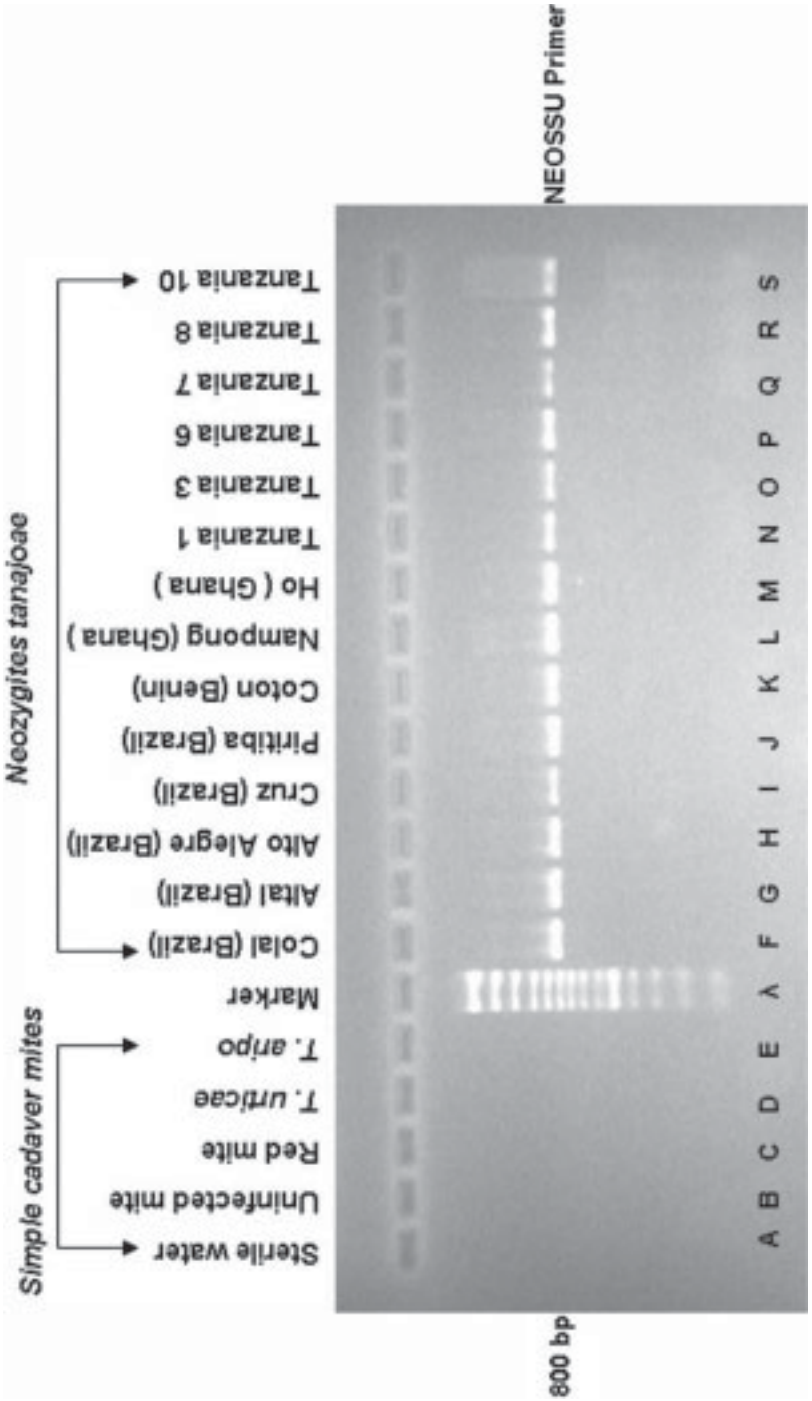

Figure 1. Detection of an 800-bp DNA fragment after polymerase chain reaction (PCR) using the oligonucleotide primers NEOSSU_F/NEOSSU_R species-specific to *Neozygites tanajoae*. Lanes F to S correspond to the amplified products while those from A to E represent the non-amplified ones. The lane λ is the marker lane. Red mite is *Oligonychus gossypii* and uninfected mite corresponds to non-infected cadaver of the cassava green mite (CGM).