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RESEARCH

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Morphological and molecular identification of *Trichoderma asperellum* isolated from a dragon fruit farm in the southern Philippines and its pathogenicity against the larvae of the super worm, *Zophobas morio* (Fabricius, 1776) (Coleoptera: Tenebrionidae)

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Abstract

Background: *Trichoderma asperellum* (Ascomycota: Hypocreaceae) is a globally recognized soil fungus due to its broad-spectrum antimicrobial and plant growth-promoting properties. To increase the availability of local strains, soil samples from a dragon fruit farm in Villanueva (Misamis Oriental, Philippines) were collected and baited using the insect-baiting technique.

Results: Using the baiting technique, *T. asperellum* strain, RMCK01, was isolated. The strain was characterized using morphological and molecular data and its biological control potential was tested using different conidial concentrations on the last larval instar of the super worm, *Zophobas morio* (Fabricius, 1776) (Coleoptera: Tenebrionidae). The ITS1 sequences of *T. asperellum* RMCK01 were 100% identical to the sequences of other *T. asperellum* isolates reported from Vietnam, India, Thailand, and China. In addition, *T. asperellum* RMCK01 was particularly efficient against *Z. morio* larvae. On day 21, 88.87%, 88.07%, and 86.73% of insects died when treated with a suspension containing 6×10^8, 3×10^9, and 2.68×10^7 conidia/ml, respectively.

Conclusion: The study highlights the potential of this fungal isolate as a biocontrol agent against insect pests.

Keywords: Entomopathogenic fungi, *Trichoderma asperellum*, Soil microorganisms, *Zophobas morio*, Biological control

Background

A major current environmental problem is caused by the indiscriminate use of pesticides under certain agricultural systems (Ortiz-Hernández et al. 2014). Pesticidal spraying can reduce or even eliminate the economic costs caused by plant diseases, insect pests, and weeds. However, there are increasing concerns regarding the overuse

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of pesticides in agriculture as they cause health problems for humans and animals and cause irreversible damage to the ecosystems. Moreover, their use is not always efficient as many pests have developed resistance against pesticides, for instance (Alvarez et al. 2017). Under these circumstances, biological control agents (BCAs) have recovered major importance. The use of beneficial microbes can, for instance, help meet the growing global demand for plant products sustainably, without relying entirely on synthetic pesticides.

Members of the fungal genus *Trichoderma* (Ascomycota: Hypocreaceae) are primarily recognized as BCAs with antagonistic effects against a wide array of fungal phytopathogens, pathogenic bacteria like *Ralstonia solanacearum*, and lepidopteran insect pests (Mohamed et al. 2020). *Trichoderma asperellum* (Samuels et al. 1999), although less studied, is also a successful BCA against many plant pathogens (Wu et al. 2017), and its larvicidal potential has been recently recognized (Podder and Ghosh 2019). Some *Trichoderma*-based products are often commercialized as BCAs against phytopathogens and as biofertilizers or growth promoters (Woo et al. 2014). The biocontrol mechanisms mainly include competition and mycoparasitism, followed by the stimulation of plant resistance and immunity (Sood et al. 2020). Amid their commercial success, a major drawback of these microbial-based fungicides is that not all strains perform the same under different environmental conditions. The collection and characterization of locally adapted strains may increase the availability of highly efficient *Trichoderma* strains (El Komy et al. 2015).

In this study, a *T. asperellum* isolate, RMCK01, was isolated, in the southern Philippines (Villaneuva, Misamis Oriental), characterized at the morphological and molecular levels. Its biological control potential was evaluated against the super worm, *Zophobas morio* (Fabricius, 1776) (Coleoptera: Tenebrionidae) larvae. To our knowledge, this is the first study to assess the biocontrol potential of *T. asperellum* against a coleopteran insect. The findings of this study pave the way and open the door to the potential application of a new bioinsecticide based on a naturally occurring entomopathogenic fungus.

Methods

Fungal isolation

The fungus was isolated from soil samples collected from a dragon fruit (*Hylocereus cactus*) farm in Villanueva, Misamis Oriental, Philippines (8.59654° N, 124.79971° E), in November 2019. To this end, the insect-baiting technique was used according to the procedure developed by Bedding and Akhurst (1975). Briefly, moistened soil samples were placed in plastic containers. Then, 5 last instar larvae, *Z. morio*, were added into each container (Navarez et al. 2021). Containers were sealed and stored in the dark at 25 ± 2 °C. Containers were inspected every other day, for 15 days, until visible symptoms of fungal infection were observed. Insect cadavers showing fungal infection were transferred to Sabouraud dextrose agar (SDA-Scharlau) media for purification (Herlinda et al. 2020). To inhibit the growth of bacteria, the media was supplemented with streptomycin, a broad-spectrum antibiotic, at a concentration of 20 µg/l (Goettel and Inglis 1997).

Morphological identification

Isolated fungi were identified based on phenotypic characteristics in SDA and PDA (Potato dextrose agar) media. Characteristics such as surface texture, surface margin, surface topography, surface pigmentation, reverse pigmentation, and growth rate were taken into account. In addition, microscopic characteristics such as the length and shape of conidia were taken into account for initial identification using the key described by Humber (1997).

Molecular identification

Genomic DNA (gDNA) of the fungal isolate was extracted using PureLink® Genomic Plant DNA Purification Kit (Thermo Fisher Scientific). Gene amplification by PCR was carried out with the following components: 3 µl of gDNA, 1 µl of ITS1 (5′tccgtaggtgaacctgcgg-3′) and 1 µl of ITS4 (5′-tcctccgcttattgatatgc-3′) primers, 2.5 µl of Taq buffer, 0.5 µl of DNA polymerase, 1 µl of dNTP mix, and 1 µl nuclease-free dH_2O. Cycling parameters on the thermal cycler were set at 95 °C for 5 min, followed by 30 cycles at 95 °C for 60 s, 58 °C for 45 s, 70 °C for 60 s, and a final extension at 72 °C for 10 min. Amplicons generated had sizes ranging from 400 to 600 bp, which was expected for ITS1 and ITS4 primers (Additional file 1: Fig. S1). Capillary sequencing was performed in a bidirectional manner at the Philippine Genome Center-Core Sequencing Facility (Manila, Philippines). Fluorescent-labeled chain terminator dNTPs with the reaction components, viz. amplicons, primers, and ABI BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific), were used. Cycling parameters on Bio-Rad T100 Thermal Cycler include: pre-hold at 4 °C; 96 °C for 60 s; 25 cycles of 96 °C for 10 s, 50 °C for 5 s, 62 °C for 4 min and hold at 4 °C. Ethanol precipitation was carried out to remove unincorporated dNTPs, excess primers, and primer dimers. Capillary electrophoresis was carried out on the ABI 3730×l DNA Analyzer using a 50 cm 96-capillary array, POP7TM Polymer, and 3730×l Data Collection Software v3.1. Base-calling was carried out on the Sequencing Analysis Software v5.4.

Sequences alignment and phylogenetic analysis

The generated sequences were manually curated and trimmed in BioEdit (Hall, 1999). The obtained sequences were searched in the National Centre for Biotechnology (NCBI) data bank using the nucleotide basic local alignment search tool (BLASTn) (Altschul et al. 1990). Sequences of *Trichoderma asperellum* RMCK01 were aligned with sequences of other related fungi species using default ClustalW parameters in MEGA 7.0 (Kumar et al. 2016) and optimized manually in BioEdit (Hall 1999), and sequences were only trimmed at both extremes after alignment to maintain fragments with similar length. Pairwise distances were computed using MEGA 7.0 (Kumar et al. 2016). Codon positions included were 1st + 2nd + 3rd + Non-coding. The base substitution model was evaluated using jModelTest 0.1.1 (Posada 2008).

The phylogenetic tree was inferred from the datasets using the Bayesian inference method (BI). All characters were treated as equally weighted and gaps as missing data. *Trichoderma longibrachiatum* (EU401556.1) and *T. brunneoviride* (EU518659.1) were used as out-groups. Bayesian phylogenetic reconstructions were performed using MrBayes 3.2.7 (Ronquist et al. 2012). The best fit model was identified as the GTR + G model test using the MrModeltest 2.0 program (Nylander 2004). Metropolis-coupled Markov chains Monte Carlo (MCMCMC) generations were run for 1×10^6 cycles, and one tree was retained every 2000 generations. The Bayesian tree was visualized using the FigTree program 1.4.4 (Rambaut 2018). In addition, genetic pairwise distances were estimated using MEGA-7 software (Kumar et al. 2016).

Mass production of spores

Mass production of spores was carried out following the method of Podder and Gosh (2019). For this, 50 g of rice was washed thoroughly under running tap water and soaked in water for 3 h. The soaked rice was transferred to a 15 × 30 cm polypropylene plastic bags and then autoclaved, maintaining a temperature of 121 °C for 15 min under 15 psi. The sterilized rice was allowed to cool down at room temperature. Then, the rice was inoculated by *T. asperellum* spores under a biological safety cabinet. *Trichoderma asperellum* spores were produced by growing the fungus on the rice. The inoculated rice was incubated at 28 ± 1 °C for 15 days (Fig. 1A).

Preparation of inoculum and quantification

Conidial concentrations were estimated based on the protocol of Goettel and Inglis (1997), with slight modifications. Briefly, mycelium was harvested, washed with distilled water, and filtered through a cheesecloth. Then, Tween 80 (0.05%) was added and 100 µl of conidial suspension was pipetted and mixed with 100 µl of sterile lactophenol blue stain solution (Barta 2010). This suspension was vortexed for 30 s and serially diluted with sterile deionized water. The number of conidia in each sample was determined using a hemocytometer (Neubauer, Marienfeld, Germany). Then, 3 conidial suspensions were prepared. Spores were counted under microscopic power of 40× and calculated using the formula (Kamaruzzaman et al. 2016):

$$\text{Total spores/ml} = \frac{\text{Number of spores counted} \times \text{dilution factor}}{\text{Number of smallest square counted}} \times 4000$$

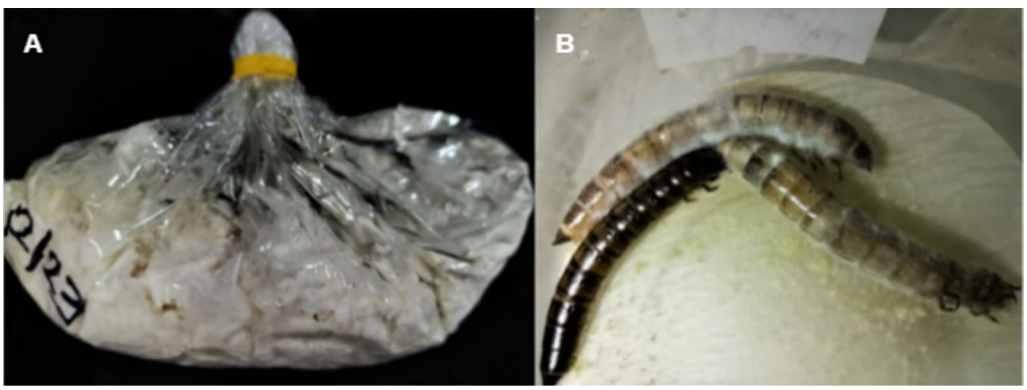

Fig. 1 Culture and biocontrol potential of *Trichoderma asperellum* RMCK01. **A** Mass production of *T. asperellum* RMCK01 using rice as culturing substrate. **B** *Zophobas morio* cadavers 3 days after spore application treatments

Finally, the suspension was standardized to 3 final concentrations using $C_1V_1 = C_2V_2$ formula.

Pathogenicity tests

To test the pathogenicity of *T. asperellum* isolate RMCK01, the last instar *Z. morio* larvae were treated with 3 conidial suspensions: 2.68×10^7, 6×10^8, or 3×10^9 conidia/ml. These 3 concentrations were selected based on previous studies (Mohamed 2019). *Zophobas morio* larvae were immersed into one conidial suspension for 60 s and then let to air dry. All insects were then transferred to 9 cm diameter clean glass Petri dishes. A piece of filter paper (9 cm in diameter) was placed at the bottom of the dish with a few drops of water. Controls were dipped into a 0.02% solution of Tween-80 prepared with distilled water. Experiments were repeated 4 times with a new batch of insects and new conidial suspensions, and for each repetition, there were 5 *Z. morio* larvae. Bioassays were incubated at 26 °C and >95% relative humidity (RH). Insect mortality and fungal infection symptoms were recorded at 12 h intervals for 21 days (Fig. 1B).

Statistical analysis

Results of statistical analysis are expressed as mean ± standard deviation ($n = 3$) with 5 replications. To determine whether there were significant differences ($p < 0.05$), one-way ANOVA was used, followed by Tukey's HSD test (XLSTAT version 2020.1.3) for post hoc analysis.

Results

Morphological characterization

Trichoderma asperellum RMCK01 mycelia were white and dark green, and arranged in 5 concentric rings, each of either white or dark green color when grown for 3 days in PDA media (Fig. 2A). When grown for 3 days in SDA media, mycelium was white with a hint of green, had a cottony texture, and forms a few concentric rings (Fig. 2B). Conidia were subglobose to globose or ovoidal (Fig. 2C, D) (Table 1).

Molecular characterization and phylogenetic relationships

Trichoderma asperellum RMCK01 had a G+C content of 56.41% and an A+T content of 43.59% in the sequences of ITS rDNA (507 bp) flanked by primers ITS1 and ITS4. These sequences were identical to other ITS rDNA sequences, available from the GenBank, of other *T. asperellum* strains isolated in Vietnam, India, Thailand, and China, suggesting that they are conspecific. The phylogenetic tree reconstructed using a Bayesian inference model supports this notion (Fig. 3). *Trichoderma asperellum* RMCK01 formed a well-supported clade with other

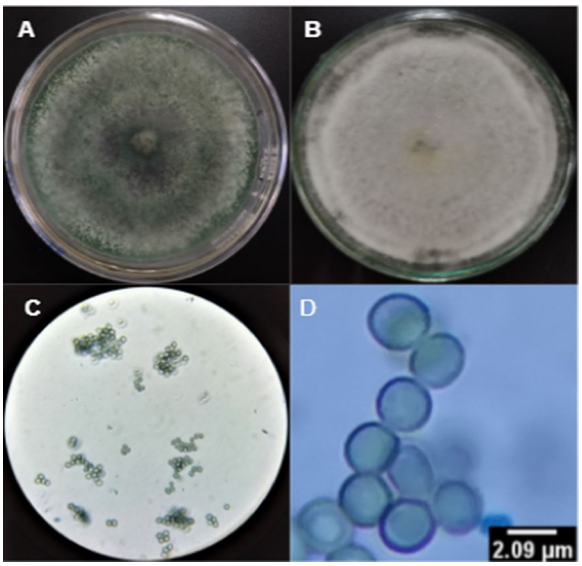

Fig. 2 Phenotypic characteristics of *Trichoderma asperellum* RMCK01: **A** Macroscopic view of *T. asperellum* cultured in Potato Dextrose Agar. **B** Macroscopic view of *T. asperellum* cultured in Sabouraud Dextrose Agar. **C** Microscopic view of the lactophenol blue mount of spores (100×). **D** Microscopic view of spores indicating their size

T. asperellum isolates from Vietnam, India, Thailand, and China. Furthermore, the phylogenetic analyses showed that *T. asperellum* was a sister group to the clade formed by *Trichoderma koningiopsis*, *T. asperelloides*, *T. viride*, *T. yunnanense*, and *T. hamatum*.

Pathogenicity of *T. asperellum* on super worm larvae

The first dead insects were observed as early as 24 h post-infection. Three days after insect death, *T. asperellum* RMCK01 sporulated, and white mycelia were observed on *Z. morio* larvae (Fig. 3). On day 7 after inoculation, significant mortality, compared to controls, was observed independently of the conidial concentration used (Fig. 4). Average mortality of larvae after 7 days ranged from 49.52 to 62.26% (Fig. 4A). On day 14 after inoculation, insect mortalities ranged between 79 and 88.07% (Fig. 4B). On day 21 post-inoculation, insect mortalities reached more than 86% (Fig. 4C). Hence, the highest mortality rate of *Z. morio* larvae by *T. asperellum* was recorded on day 21 with a suspension of 6×10^8 (88.87%), followed by 3×10^9 (88.07%) and the least infection was from 2.68×10^7 conidia/ml (86.73%).

Discussion

Use of *Trichoderma,* as a biocontrol agent against insect pests, has been studied in recent years, uncovering this group of fungi as a promising alternative to be used in

Table 1 Summary of morphological characterization of *Trichoderma asperellum* RMKC01 isolate from a dragon fruit farm in Villanueva, Misamis Oriental, Philippines

Species	Culture media	Length of conidia	Shape of conidia	Surface texture	Surface margin	Surface topography	Surface pigmentation (3 days)	Reverse pigmentation (3 days)	Growth rate
Trichoderma asperellum	PDA	1.5–2.9 um	Globose	Wooly	Curled	Flat	White/dark green	White/dark green	5.2 cm (3 days)
	SDA		Globose	Wooly	Curled	Flat	white/light Green	White/light green	5.7 cm (3 days)

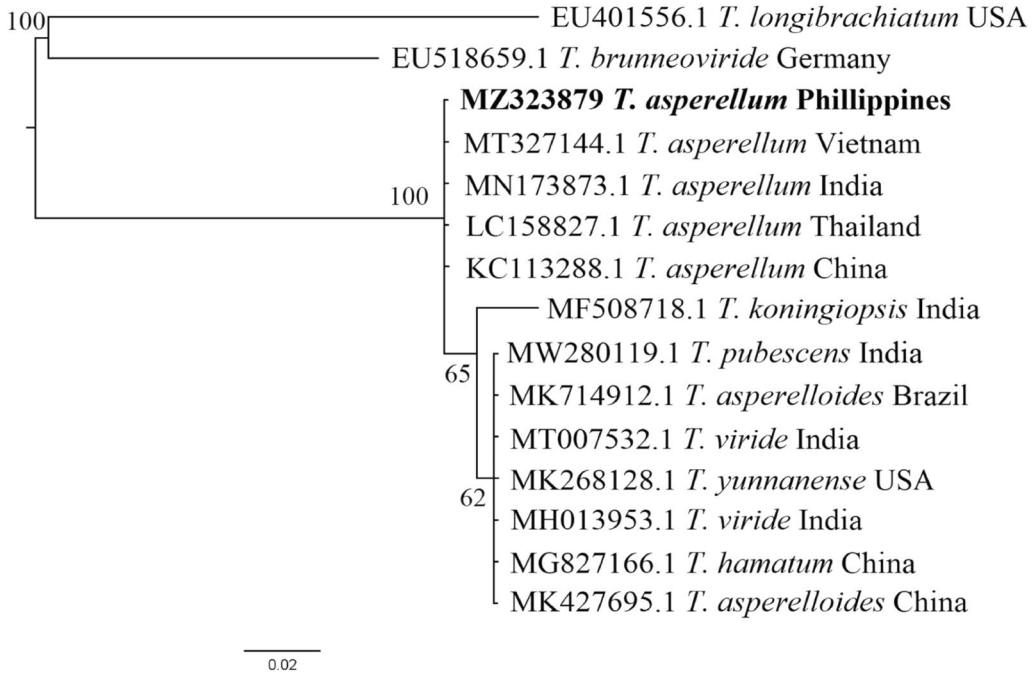

Fig. 3 Phylogenetic relationships of *Trichoderma asperellum* and other *Trichoderma* species as inferred from Bayesian analysis of sequences of the Internal Transcribed Spacer rDNA region. Bayesian posterior probabilities (%) equal to or more than 60% are given. The scale bar shows the number of substitutions per site

sustainable agriculture not only because of its efficiency against plant pathogens but also insect pests (Poveda 2021). The reports of its economic importance in the agricultural industry and the characteristics of *Trichoderma* that produces large amounts of fungal spores make it ideal for inoculum production under laboratory conditions (Herrera-Téllez et al. 2019). In this study, evidence that *T. asperellum* naturally occurs in agricultural soils in the Philippines was presented and it can be grown under laboratory conditions and is highly pathogenic against *Z. morio* larvae.

Several studies show the biological control potential of *T. asperellum* against plant diseases (Vilcacundo et al. 2020); at the same time, it improves plant growth and development (Moreira et al. 2021) in several plant species and also bioremediation qualities are shown (Li et al. 2021). Furthermore, a more recent study reports that this fungal species can have larvicidal effects against *Anopheles* spp., the vector of malaria and *Aedes aegypti* (da Silveira et al. 2021). The potential of *T. asperellum* to control other insect pests has not been shown yet. Hence, to determine the potential of *T. asperellum* and to control other insect pests, its pathogenicity against *Z. morio* was evaluated under laboratory conditions. The present experimental results showed that *T. asperellum* strain RMKC had also a great potential to control *Z. morio* larvae as the insect mortality was observed a few days after fungal applications, which reached about 86–88% within

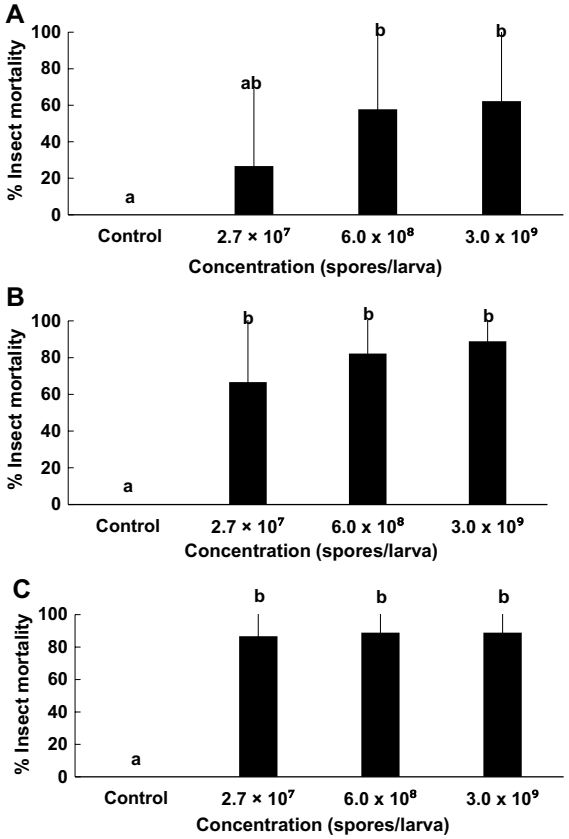

Fig. 4 Pathogenesis of *Trichoderma asperellum* RMCK01 against *Zophobas morio*. Average mortality of last instar larva of *Z. morio* after: **A** 7 days, **B** 14 days, and **C** 21 days of applying *T. asperellum* spores. Error bars indicate standard deviations of four replicates with five larvae each. Different letters above error bars indicate significant differences between treatments (Tukey test; $p \leq 0.05$)

21 days after treatments. The presented results indicate a considerable potential for use of *T. asperellum* as an entomopathogenic fungus to control insect pests with the suspension even as low as 2.7×10^7 conidia/ml. Notwithstanding the present results, additional experiments to focus on long shelf life, ease of application, pathogen and pest virulence, and a wider spectrum of action and yield assessments must be conducted before ultimate conclusions are presented. Furthermore, a technology for co-cultivation of 2 microbes, in particular, *T. asperellum* GDFS1009 and *B. amyloliquefaciens* 1841 (Karuppiah et al. 2019), and *T. asperellum* and *Bacillus subtilis* (Senkovs et al. 2021) has been recently reported to significantly enhance the plant growth and protection against plant pathogens. Thus, to utilize its various benefits, co-cultivation with other microbes is also recommended for further study of *T. asperellum.*

Conclusions

Trichoderma asperellum RMKC01, isolated from an agricultural soil, was highly pathogenic against the larvae of the super worm, *Z. morio*. Further studies to evaluate its pathogenicity against other insect pests and non-targeted organisms are required to assess its real potential to be used as a biocontrol agent in organic and eco-friendly agricultural settings.

Abbreviations

BCA: Biological control agent; gDNA: Genomic DNA; PDA: Potato dextrose agar; SDA: Sabouraud dextrose agar; *T. asperellum*: *Trichoderma asperellum*; *Z. morio*: *Zophobas morio*.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41938-022-00548-0.

Additional file 1: Fig. S1. Amplicon products at 1% agarose with the DNA marker (1 Kb plus ladder, Invitrogen). Amplicons showed the good quality of single and intense band for each sample with 400–600 bp size target, were purified using Exosap IT to remove the unnecessary smears and other possible inhibitors.

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Author contributions

RS, KRG, MS, CJA, and NHS conceptualized and designed the experimental work. RS, KRG, MS, CJA, PN, and NHS provided material. RS, KRG, MS, and CJA performed the field and laboratory experiments, RS collected and analyzed the data, under the supervision of NHS and advice of PN, AHB, and RARM. NHS was the in-charge of resources and supervision. RS wrote the manuscript together with NHS and with substantial input of all other authors. All authors agreed with the submission of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All available data are included in this manuscript. The DNA sequence data have been submitted to the NCBI GenBank data repository.

Declarations

Ethics approval and consent to participate

This research does not contain human participants.

Consent for publication

Not applicable.

Competing interests
The authors declare that they have no competing interests.

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