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Molecular Characterization and Ecological Interactions of Fungal Pathogens Associated with *Carcinopsis* sp. Wētā in the Dogny Forest, New Caledonia

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Abstract

As natural regulators of insect populations, entomopathogenic fungi (EPF) can affect native species. This research investigates the infection of *Carcinopsis* sp. wētā by an entomopathogenic fungus in the Dogny Forest, New Caledonia, and explores associated ecological dynamics. Molecular characterization revealed that the fungus associated with the wētā belongs to the entomopathogenic *Beauveria malawiensis*. A potential secondary pathogen, *Trichoderma uncinatum*, was also isolated and grown on a culture medium. The diversity highlights the complex interactions between fungi and their hosts. The presence of these entomopathogenic fungi on wētā raises concerns about their potential future use as bioagents against pests. Monitoring and regulating the transfer of insect materials between the New Caledonia and New Zealand archipelagos are crucial for wētā conservation efforts. Further research is needed to better understand the ecological interactions between wētā and fungal pathogens, as well as their implications for forest ecosystem dynamics. This study underscores the importance of proactive conservation measures to preserve biodiversity in both archipelagos.

Keywords – *Beauveria malawiensis* – conservation – entomopathogens – *Trichoderma uncinatum* – wētā

Introduction

Entomopathogenic fungi (EPF) are a group of fungi that infect and kill insects, playing a critical role in regulating insect populations and offering potential as biological control agents (Imoulan et al. 2017, Perumal et al. 2024, Quesada-Moraga et al. 2024). These fungi have been studied extensively for their ability to target pest species, reducing the reliance on chemical pesticides (Deaver et al. 2019). However, there are significant gaps in our understanding of the geographic and taxonomic specificity of these fungi, particularly in unique and isolated ecosystems such as New Caledonia.

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The biological diversity encountered in New Caledonia is remarkable (Ibanez et al. 2014, Carriconde et al. 2019, Pillon et al. 2021). Even though, there are few local studies (Cochereau et al. 1995 for a local study of *Beauveria bassiana*), the diversity and distribution of entomopathogenic fungi in this region remain largely unexplored. For instance, *Beauveria bassiana* and *Metarhizium anisopliae* have been widely studied and utilized (Faria & Wraight 2007, Mauchline & Stannard 2013), however, their distribution and diversity in New Caledonia are poorly documented (Cochereau et al. 1995). Additionally, the fungal-insect interaction in this region is poorly understood (Hajek & St Leger 1994, Boomsma et al. 2014). There is also a paucity of data on the specificity of fungal strains on different insect hosts. For example, certain strains of *Metarhizium anisopliae* exhibit high virulence towards pest insects such as *Zeugodacus cucurbitae* (Diptera, Tephritidae), *Microtermes obesi* (Blattodea, Termitidae), and *Scolytus scolytus* (Coleoptera, Curculionidae) in other climates (Onsongo et al. 2019, McGuire & Northfield 2020), while the virulence of other EPF against endemic insects in New Caledonia is not yet known. This gap in taxonomic specificity highlights the need for targeted research to identify and characterize fungal strains that are adapted to the region's unique ecological conditions and insect biodiversity (Onsongo et al. 2019, McGuire & Northfield 2020). Addressing these gaps is crucial for harnessing entomopathogenic fungi as bioagents, ensuring their efficacy and sustainability in pest management strategies and protecting biodiversity.

The introduction of entomopathogenic fungi into new environments, particularly through intentional releases for pest control purposes, can pose risks of unintended establishment and spread (Tahira et al. 2014). It is important to carry out studies to identify a fungal agent specific to the pests targeted, while ensuring that it does not affect local and sensitive fauna. Entomopathogenic fungal preparations are already being used as bioagents against pests (Brownbridge et al. 2010, Mauchline & Stannard 2013) in New Zealand, a biogeographic partner of New Caledonia (Trewick et al. 2007). Therefore, it is important to accurately identify these pathogens to avoid their misuse as biocontrol agents, particularly when transferring insect-type materials between New Caledonia and New Zealand, especially if the strain is not naturally found in both regions.

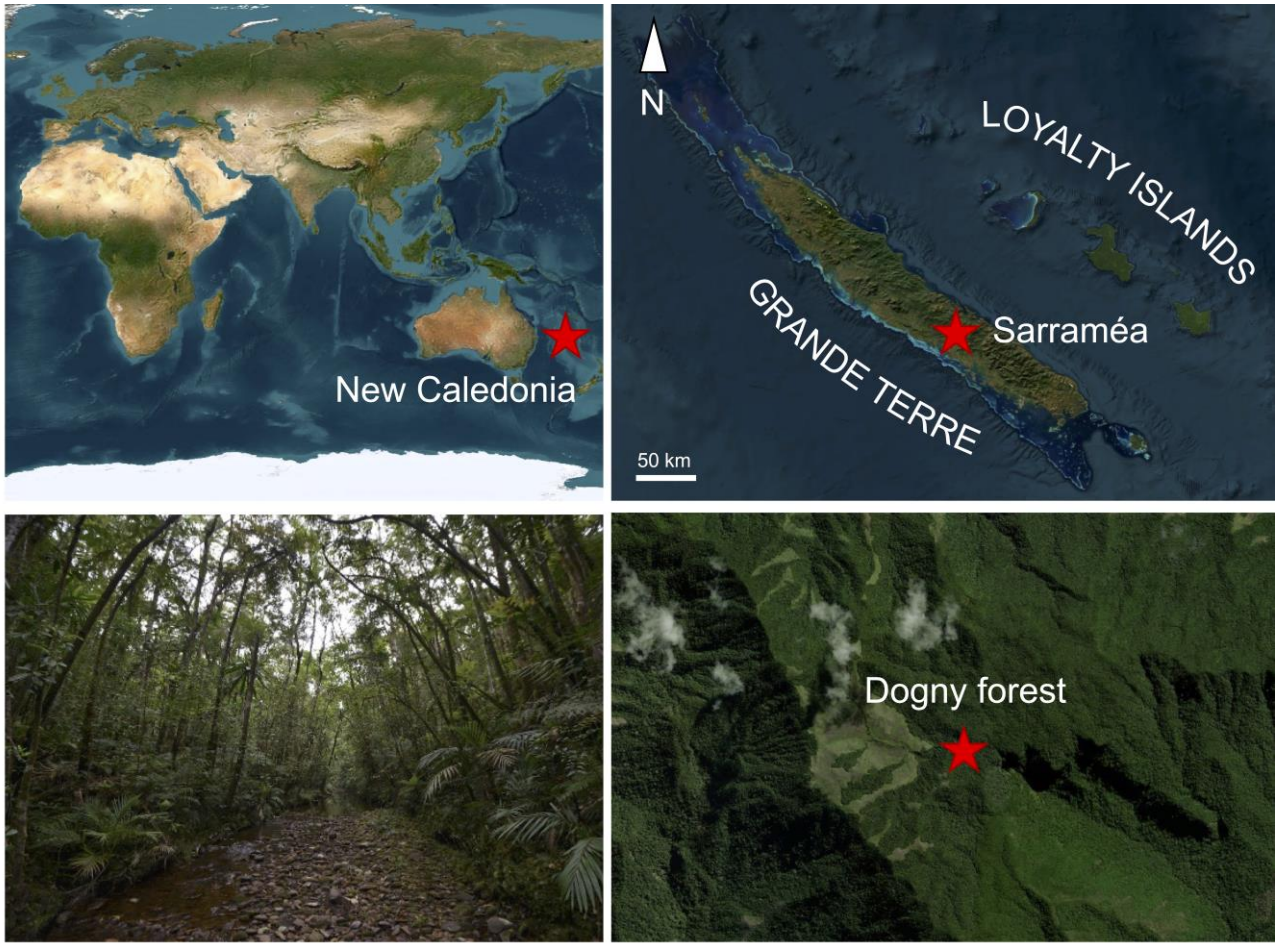
This study involves identifying fungal pathogens newly found in a *Carcinopsis* wētā (Orthoptera, Anostostomatidae) in the Dogny Forest (Sarraméa, New Caledonia) based on molecular analyses. Ecological interactions occurring on the dying wētā are reported, indicating potential infection pathways. Among the New Caledonian wētā, the genus *Carcinopsis* is a large, flightless insect endemic to the archipelago, often characterized by their robust exoskeletons and long antennae. Despite their probable ecological significance, the *Carcinopsis* genus remains largely unstudied, with little known about their biology, behaviour, or distribution; moreover, no attacks by entomopathogenic fungi have been reported so far. Anostostomatidae taxa are valuable examples of a faunal lineage shared with New Zealand (Pratt et al. 2008, Trewick et al. 2024), enabling ecological comparisons (Dowle et al. 2015, Quenu et al. 2023, Platania et al. 2024). *Carcinopsis* Brunner in New Caledonia is most closely allied to *Motuweta* Johns in New Zealand. So far, no entomopathogenic fungi have been reported in *Motuweta*. This probably reflects the lack of observations, even though similar infections have been found in related wētā species like *Hemideina* Walker and *Deinacrida* White (Glare et al. 1993).

Materials & Methods

1. Samples collection and isolation

Fieldwork was conducted on November 12, 2021, at 8:00 PM in the Dogny Forest (GPS coordinates: 21°37'04.8"S 165°53'09.9"E), Sarraméa County, New Caledonia (Fig. 1), where a *Carcinopsis* sp. wētā with signs of fungal infection was found on a tree trunk. The study of ecological interactions (predation, necrophagy, host viability) continued until November 13, 2021. After three days of ongoing monitoring, the wētā was eventually collected in a zip lock bag at 8:00

PM. *In situ* and laboratory pictures were captured with a Nikon D800, an SB800 flash and a homemade light diffuser.



Figs 1 – Location of New Caledonia archipelago (top left), and of the Sarraméa County on the Grande Terre Island (top right). Precise location in the Dogny Forest where the wētā was collected (bottom right), and a picture of the wētā's biotope in this place (bottom left). USGS/NASA Landsat satellite image, picture is copyright of Pierre-Louis Stenger.

On November 16, 2021, a small piece of infected wētā was put in cetyltrimethylammonium bromide (CTAB 2X) for future extraction. Potato Dextrose Agar (PDA) was prepared according to standard protocols (39 g/L of distilled water). The mixture was heated to boil while being stirred continuously. After boiling, the PDA solution was sterilized in an autoclave at 121°C for 15 minutes. Once sterilized, the solution was poured onto sterile Petri dishes to cool and solidify. Fungal isolates obtained from the infected *Carcinopsis* wētā were cultured onto PDA plates using a sterile inoculation loop, by placing infected tissue directly on the medium. The inoculated PDA plates were then sealed with parafilm and incubated in a dark incubator at 28°C. Plates were monitored regularly for fungal growth and development.

2. DNA extraction and molecular analysis

DNA was extracted directly from the infected wētā for fungal and host identification, and from fresh fungal mycelia grown on PDA. DNA was extracted using the Wizard genomic DNA purification kit (Promega) according to Carriconde et al. (2008). DNA concentration was measured with a NanoDropTM (Thermo Fisher Scientific, Waltham, MA, USA) and DNA quality was verified by electrophoresis in a 1% Agarose gel. For fungal identification, the internal transcribed spacers (ITS) of the 45S ribosomal RNA cassette were amplified using the primers ITS1-F and ITS4

(White et al. 1990, Gardes & Bruns 1993). For the insect, the partial mitochondrial DNA cytochrome c oxidase I (COI) gene was amplified using the LCO1490 and HCO2198 primers (Folmer et al. 1994). Sequencing was carried out using the primers mentioned above.

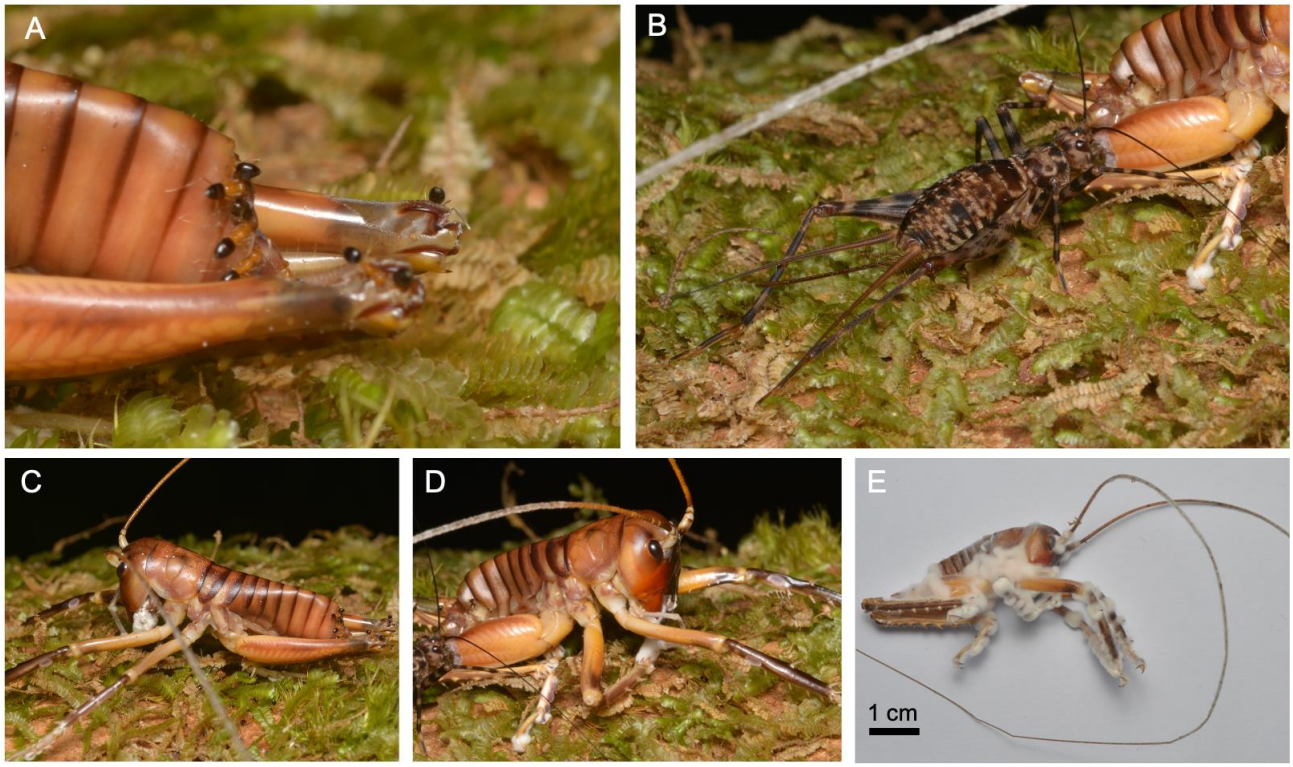
Data preprocessing was carried out using the BioEdit software (V. 1.0) (Hall 1999). Raw chromatograms and sequences were verified for error corrections. Poor-quality regions at the beginning and end of chromatograms were identified and trimmed manually, and misread bases were modified accordingly. The corrected consensus sequences were subjected to BLAST searches in the GENBANK database (Benson et al. 2013). Sequences of closely related species based on the order of the BLAST top hits on NCBI (Table 1) were retrieved to construct phylogenetic trees. Multiple sequence alignments (MSA) of the ITS sequences were performed using ClustalW (Thompson et al. 2003), implemented in the MEGA software (v.11) (Tamura et al. 2021). Alignment parameters were optimized according to recommendations from Hall (2013) and Dissanayake et al. (2020), ensuring accuracy for fungal sequences. Post-alignment adjustments, including the removal of ambiguously aligned regions, were performed to refine the dataset. Maximum Likelihood (ML) phylogenetic trees were constructed in MEGA (v.11) following the methodology described by Hall (2013) and Dissanayake et al. (2020). The best-fit substitution model for each alignment was determined using the model selection tool in MEGA, based on the Akaike Information Criterion (AIC). Bootstrap analysis with 1,000 replicates was employed to evaluate the robustness of tree topologies. The resulting phylogenetic trees were exported and visualized in R using the *Ggtree* package (Yu et al. 2017) to enhance graphical representation and facilitate interpretation. The corrected consensus sequences were deposited in the GenBank database with the following accession numbers: PP786545 for the wētā, PP784310 for *Beauveria malawiensis* and PP784311 for *Trichoderma uncinatum***.**

Results

Scavenging by *Paraparatrechina foreli* ssp. *nigriventris* ants (Fig. 2A) were observed on the first day and scavenging by either the genus *Gryllodes* or *Agnotecous* (Fig. 2B) was observed on the second day in situ. On the first day, fungal hyphae were visible on the front legs, maxilla, jaws, palps of the accessory jaw and palp of the lower lip of the wētā. At this point, the wētā still showed slight movement. The next day, the wētā had moved only a short distance on the trunk (about 30 centimetres). Fungal growth spread to the wētā's tarsi and tibia, and subsequently expanded in surface area in parts that had already been infected. Three days later after collecting the infected wētā, fungal growth proliferated to half of the insect from the base of the antennae to the spiracles, covering the legs and its entire underside.

Using the NCBI BLAST tool to analyze the insect DNA sequence did not yield a close match due to the lack of sequence data for the latter. However, alignment and comparison with unpublished mtDNA COI sequences from Anostostomatidae gave conclusive support for the genus *Carcinopsis*, though not for a specific species.

Analysis of the fungal pathogens revealed the presence of *Beauveria malawiensis* on the wētā and *Trichoderma uncinatum* on PDA. Indeed, the ITS sequences of *Beauveria malawiensis* (LC768997.1) from the wētā exhibited 100% coverage (548 bp) and 99.45% homology, while those of *Trichoderma uncinatum* (MK795994.1) from the PDA culture exhibited 100% coverage (582 bp) and 99.49% homology (Table 1, Fig. 3). Sequences of *B. malawiensis* and *B. brongniartii* strains (the second phylogenetically closest hit) were used for the phylogenetic tree construction (Figs. 3). The *Beauveria* sp. from Dogny Forest clusters with all *B. malawiensis* strains and one strain of *B. brongniartii* (Table 1). For the PDA culture, only one sequence of *T. uncinatum* was found on NCBI (from the holotype), and the second phylogenetically closest species was *T. viride*, so additional strains from this species were added (Table 1). The *Trichoderma* sp. from Dogny Forest is phylogenetically close to *T. uncinatum* (Figs. 3).



Figs 2 – A Scavenging of the infected *Carcinopsis* wētā by *Paraparatrechina foreli* ssp. *nigriventris* ants in Dogny Forest, New Caledonia. B Scavenging by a cricket of either the genus *Gryllodes* or *Agnotecous* on the infected wētā. C First day (November 12), second day (November 13). D and fifth day (November 16). E of observed fungal growth. Pictures are copyright of Pierre-Louis Stenger.

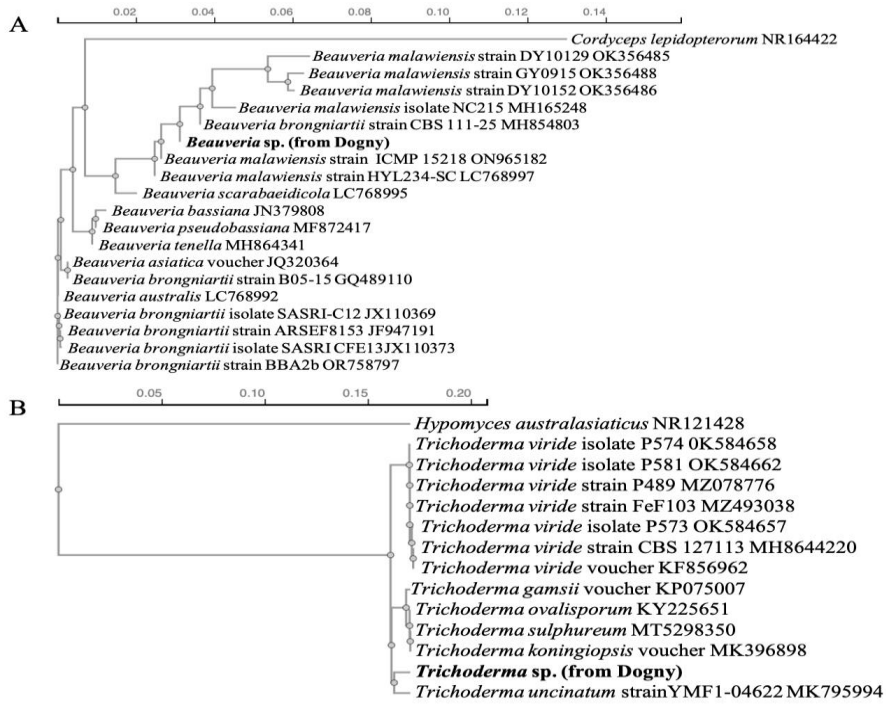


Fig. 3 – Maximum likelihood phylogenetic trees of A *Beauveria* from the wētā. B *Trichoderma* from the PDA culture exhibited with top hit NCBI blast species from Table 1.

Table 1 Top hit NCBI blast species results from the sequences from the wētā and the PDA culture used for Figs 3.

Organism	**Accession Number**	**Source/Host**	**Location**
Beauveria asiatica voucher	JQ320364	Soil sample	China, Fuzhou, Fujian
Beauveria australis	LC768992	Single conidium from unknown insect carcass	Taiwan
Beauveria bassiana	JN379808	Scarabaeidae larvae	Mexico
Beauveria brongniartii isolate SASRI CFE13	JX110373	*Hypopholis sommeri* beetle (Coleoptera: Scarabaeidae)	South Africa
Beauveria brongniartii isolate SASRI-C12	JX110369	*Galleria mellonella* in soil (Lepidoptera: Pyralidae	South Africa
Beauveria brongniartii strain ARSEF8153	JF947191	*Agrilus planipennis* (Coleoptera: Buprestidae)	Canada
Beauveria brongniartii strain B05-15	GQ489110	Unknown host	China
Beauveria brongniartii strain BBA2b	OR758797	Unknown host	Czech Republic
Beauveria brongniartii strain CBS 111-25	MH854803	Unknown host	Unknown
Beauveria malawiensis isolate NC215	MH165248	*Vespula vulgaris* (Hymenoptera: Vespidae)	New Zealand
Beauveria malawiensis strain DY10129	OK356485	Unknown beetle (Coleoptera)	China
Beauveria malawiensis strain DY10152	OK356486	Unknown beetle (Coleoptera)	China
Beauveria malawiensis strain GY0915	OK356488	Unknown ladybug (Coleoptera: Coccinellidae)	China
Beauveria malawiensis strain HYL234	LC768997	Unidentified Cerambycinae (Coleoptera)	Taiwan
Beauveria malawiensis strain ICMP 15218	ON965182	Lepidoptera (caterpillar)	New Zealand, Waitakere Ranges
Beauveria pseudobassiana	MF872417	*Hylastinus obscurus* found in soil (Coleoptera: Curculionidae)	USA, Oregon
Beauveria scarabaeidicola	LC768995	Stroma with teleomorph	Taiwan
Beauveria tenella	MH864341	Unknown host	USA, Wyoming
Cordyceps lepidopterorum	NR164422	Culture from holotype of *Cordyceps lepidopterorum*	Thailand
Hypomyces australasiaticus	NR121428	Culture of *Hypomyces australasiaticus*	Estonia
Trichoderma gamsii voucher	KP075007	Leaf litter	Viet Nam
Trichoderma koningiopsis voucher	MK396898	Blackberry (*Rubus* sp.) roots (Plantae, Rosaceae)	Mexico
Trichoderma ovalisporum	KY225651	Isolated from soil	China
Trichoderma sulphureum	MT529835	Unknown host	China
Trichoderma uncinatum	MK795994	Holotype of *Trichoderma uncinatum*	China

Table 1 Continued

Organism	Accession Number	Source/Host	Location
Trichoderma viride	MH864422	Culture (unknown)	New Zealand
Trichoderma viride isolate P573	OK584657	Forest soil, relict charcoal hearth	Poland, Manowo Forest District
Trichoderma viride isolate P574	OK584658	Forest soil, relict charcoal hearth	Poland, Manowo Forest District
Trichoderma viride isolate P581	OK584662	Forest soil, relict charcoal hearth	Poland, Manowo Forest District
Trichoderma viride strain FeF103	MZ493038	*Fraxinus excelsior*, dead leaf petiole (Plantae, Oleaceae)	Poland, Dynów
Trichoderma viride strain P489	MZ078776	*Quercus robur*, seedling's root (Plantae, Fagaceae)	Poland, Kalina-Lisiniec
Trichoderma viride voucher	KF856962	*Tsuga canadensis*, in roots (Plantae, Pinaceae)	USA, Tennessee

Discussion

The description of the ecological interactions *in situ* indicates that despite the fungal infection, the wētā appears to be alive as it was able to move along the trunk over 24 hours. This suggests that the fungus has possibly taken control over the insect to position it higher off the ground, similar to how ants infected by the entomopathogen *Ophiocordyceps unilateralis* behave (De Bekker et al. 2014). Such behaviour has also been observed in other parasitic organisms that induce a 'zombification' effect on their hosts (Harmon 2012). Alternatively, this wētā may not be an optimal host for the identified *Beauvaria* species, and further research is needed to clarify the host specificity of entomopathogenic fungal strains (Imoulan et al. 2017, Rohrlich et al. 2018). During the fungal infection, various other species, including ants and crickets took advantage of the partial immobilisation of the wētā to feed on the infected host. This raises questions about the host specificity of this and other entomopathogenic fungi in New Caledonia, as well as the potential for transmission from one insect host to another. While the diet of *Carcinopsis* is not well known, if it resembles related species in New Zealand, it is likely predatory and may become infected with pathogenic fungi by scavenging on dying insects.

Molecular characterization of the fungal pathogens offers valuable insights into the diversity and composition of fungal communities associated with insects in Dogny Forest and more broadly in New Caledonia. DNA sequence data indicated that the fungus infecting the *Carcinopsis* wētā is a species of *Beauveria*, most likely *B. malawiensis. Beauveria malawiensis* is an entomopathogenic species known to infect New Zealand Coleoptera, Hymenoptera and Hemiptera (Cummings 2009), but there are no records from wētā or other Orthoptera. Some *Beauveria* species have been extensively studied as biocontrol agents and form the basis for several commercially available mycoinsecticides (Faria & Wraight 2007). For instance, preparations of *Beauveria bassiana* are used in New Zealand as bio-insecticides against *Bactericera cockerelli* (Hemiptera) (Mauchline & Stannard 2013).

Monitoring entomopathogens in New Caledonia, particularly in infected insect species, would be beneficial to identify fungal species that could be multispecific and to prevent their excessive use as biocontrol agents in agriculture (e.g. Tahira et al. 2014), especially in the context of reducing the use of chemical products. It is recommended to be cautious in developing and applying entomopathogens without a thorough assessment of their potential impacts on native insect populations. At this point, it would be appropriate to avoid using *B. malawiensis* as a treatment for pests in either archipelago as this could affect the endemic insect populations, including Anostostomatidae (wētā). However, a highly virulent strain of *B. bassiana* was

discovered several years ago in New Caledonia on the coffee berry borer, *Hypothenemus hampei* (Cochereau et al. 1995), and its DNA sequence is more distant from the *Beauveria* species found in the Dogny Forest (Figs 3). This strain could be a candidate for cultivation and multiplication studies to produce a local bioinsecticide once further analysis is done. Recently, a strain of *B. bassiana* has also been isolated from an unidentified caterpillar in New Caledonia (F. Carriconde & C. Mille, pers. com.).

The fungus grown on PDA was identified as *Trichoderma uncinatum*. *Trichoderma* species are entomopathogens of pest insects, including *Leucinodes orbonalis* (Lepidoptera) and *Macrosiphum euphorbiae* (Hemiptera) (Ferreira & Musumeci 2021). Moreover, *Trichoderma* species are fungicidal and nematicidal agents, as well as plant pathogens for some species (Ferreira & Musumeci 2021). Thus, this multipathogen species appears to be an opportunistic and secondary pathogen.

It is noteworthy that sequencing the fungi on the wētā identified an entomopathogen (*Beauveria malawiensis*) while that grown on PDA yielded a multipathogen (*Trichoderma uncinatum*). This suggests that entomopathogenic infections could involve a community of species rather than a single species functioning alone. In addition, this could be due to the fact that we did not perform single spore isolation or hypahale isolation. However, we cannot exclude the possibility that the development of the *Trichoderma* species could be a consequence of infection rather than being directly linked to it. On the wētā, the abundant fungus corresponds to an environment conducive to its growth (the insect) whereas the PDA medium provides a more suitable environment for another species, making it a phytopathogen. Different culture media are optimal for *Beauvaria* fungi. This complex diversity highlights the importance of using Next Generation Sequencing methods such as meta-barcoding when studying entomopathogens, especially in scenarios where several organisms are involved and traditional culture techniques fall short (Francisco et al. 2006, Deaver et al. 2019).

In New Caledonia, the systematics and ecology of wētā are poorly known and less studied compared to their relatives in New Zealand. A comparative investigation of the anostostomatid fauna in these two archipelagos is in progress (Trewick & Morgan-Richards 2004, 2005, Trewick et al. 2024, SA. Trewick unpublished). This also underscores the importance of biosecurity measures to manage and monitor the exchange of insect materials between the two regions, whether for biodiversity assessments or commercial purposes. Further research is needed to clarify the specific mechanisms underlying the observed ecological interactions and to evaluate the impacts of fungal pathogens on *Carcinopsis* wētā populations and other native insects, as well as their roles in forest ecosystem dynamics. Such studies are essential for understanding the resilience of forest ecosystems to environmental changes, insect populations, and fungal life cycles, and to design conservation strategies aimed at preserving biodiversity in New Caledonia and New Zealand's forests.

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