# Filamentous Fungi Isolated in Simuliidae (Diptera: Nematocera) larvae in Brazilian Amazônia

# Fungos Filamentosos Isolados do trato digestivo de Larvas de Simuliidae (Diptera: Nematocera) da Amazônia brasileira

DOI: 10.34188/bjaerv6n4-020

Recebimento dos originais: 05/08/2023
Aceitação para publicação: 30/09/2023

**Yamile Benaion Alencar**
Doutora em Entomologia, Instituto Nacional de Pesquisas da Amazônia/Universidade Federal do Amazonas
Instituição: Instituto Nacional de Pesquisas da Amazônia, Coordenação de Pesquisas em Entomologia
Endereço: Avenida André Araújo, 2936, Petrópolis, 69011-970, Manaus- AM, Brasil
E-mail: yamile.alencar@gmail.com

**Neusa Hamada**
Doutora em Entomologia pela Clemson University, Estados Unidos.
Instituição: Instituto Nacional de Pesquisas da Amazônia, Coordenação de Pesquisas em Entomologia
Endereço: Avenida André Araújo, 2936, Petrópolis, 69011-970, Caixa Postal 478, Manaus, AM, Brasil
E-mail: nhamada@inpa.gov.br

**ABSTRACT**
The objective of this study was to isolate and identify fungal species found on Simuliidae larvae in several municipalities of Brazilian Amazonia, especially in the states of Amazonas and Rondônia. The larvae were collected using forceps, pipettes, placed in sterile containers and stored under refrigeration before being dissected in the laboratory. Approximately 30 Simuliidae species were macerated in Saline Solution (0.9%), seeded on Petri dishes containing solid culture media. The total of 87 fungal lineages were identified from homogenates of seven black-fly species: *Simulium perflavum* Roubaud, *S. trombetense* Hamada, Py-Daniel & Adler, *S. maroniense* Floch & Abonnenc, *S. cauchense* Floch & Abonnenc, *S. daltanhani* Hamada & Adler, *S. rorotaense* Floch & Abonnenc and *Lutzimulium simplicicolor* (Lutz). Fifteen fungal species were identified as: *Cladosporium chlorocephalum* (Fresen.) (1.1%), *C. herbarum* (Pers.) Link (1.1%), *Penicilium crustosum* Thom (2.2%), *P. chrysogenum* Thom (3.4%), *P. lividum* Westling (3.4%), *P. citrinum* Thom (5.7%), *P. citreonigrum* Dierckx (1.1%), *P. corylophilum* Dierckx (1.1%), *P. fellutanum* Biourge (11.4%), *P. oxalicum* Currie and Thom (2.2%), *Gliocladium virens* Corda (5.7%), *Aspergillus japonicus* Saito (10.3%), *Trichoderma harzianum* Rifai (10.4%), *T. koningii* Oud. Aggr. (4.6%), *Pestalotiopsis guepini* (Desmazieres) Steyaert (12.6%) and *Mycelia sterilia* (22.9%). Knowledge about these fungi and their relationship with Simuliidae larvae can contribute to their use in biological control of disease vectors and to their use in biotechnological studies, with fungal lineages selected for economic potential.

**Keywords:** Insecta, anamorphic fungi, isolation, endosymbiosis

**RESUMO**
O objetivo deste estudo foi isolar e identificar espécies de fungos em larvas de Simuliidae em vários municípios da Amazônia, especialmente nos Estados do Amazonas e Rondônia. As larvas foram coletadas usando-se pinças, pipetas, colocadas em recipientes plásticos estéreis e conservadas sob refrigeração antes de serem dissecadas no laboratório. Aproximadamente, 30 larvas de Simuliidae de cada espécie foram maceradas em Solução Salina (0,9%) e semeadas em placas de Petri contendo meio de cultura sólido. O total de 87 linhagens fúngicas foi identificado no macerado de larvas de sete espécies de Simuliidae: *Simulium perflavum* Roubaud, *S. trombetense* Hamada, Py-Daniel & Adler, *S. maroniense* Floch & Abonnenc, *S. cauchense* Floch & Abonnenc, *S. daltanhani* Hamada & Adler, *S. rorotaense* Floch & Abonnenc e *Lutzimulium simplicicolor* (Lutz). Quize espécies de fungos foram identificadas como: *Cladosporium chlorocephalum* (Fresen.) (1.1%), *C. herbarum* (Pers.) Link (1.1%), *Penicilium crustosum* Thom (2.2%), *P. chrysogenum* Thom (3.4%), *P. lividum* Westling (3.4%), *P. citrinum* Thom (5.7%), *P. citreonigrum* Dierckx (1.1%), *P. corylophilum* Dierckx (1.1%), *P. fellutanum* Biourge (11.4%), *P. oxalicum* Currie and Thom (2.2%), *Gliocladium virens* Corda (5.7%), *Aspergillus japonicus* Saito (10.3%), *Trichoderma harzianum* Rifai (10.4%), *T. koningii* Oud. Aggr. (4.6%), *Pestalotiopsis guepini* (Desmazieres) Steyaert (12.6%) e *Mycelia sterilia* (22.9%). O conhecimento sobre esses fungos e suas relações às larvas de Simuliidae pode contribuir para o uso em controle biológico de insetos vetores e também em estudos biotecnológicos, com a seleção de linhagens com potencial econômico.

**Palavras-chave:** Insecta, fungos anamorfos, isolamento, endosimbiose

## 1 INTRODUCTION

The family Simuliidae (Insecta: Diptera), or black flies has a worldwide distribution. In Brazil the main human disease transmitted by black flies is onchocerciasis (river blindness). The bites of many species in this family are painful to humans and in some areas of southern Brazil they have been a serious problem affecting rural communities, decreasing agricultural productivity and tourism (Souza 1984). The females of these insects are, in general, hematophagous, being potential vectors of viruses, bacteria, protozoa and helminthes.

Microorganisms can be present in the external surface of the larval body or internally, associated with the digestive tract, they can have complex relationships with their hosts, and in the majority of the cases this relationship is beneficial to the insect and/or to the microorganism (Alves 1998).

Worldwide, many publications report interactions from pathogenic to obligate mutualism between fungi and insects (Yeboah et al. 1984, Lichtwardt 1986, 1994, Golkar et al. 1993, López Lastra & García 1997, Lichtwardt et al. 2000, McCreadie & Adler 2005, Scholte et al. 2004, Sosa-Gómez et al. 2010). In Brazil, there are many studies of relationships between fungi and insects (Moraes et al. 1998, 1999, Arantes & Correia 1999, Moraes et al. 2001, Sales et al. 2002), but especially in Amazonia the references with aquatic insects are scarce (Ríos-Velásquez & Hamada 2002, Alencar et al. 2003, Pereira et al. 2005, Alencar et al. 2017).

In this study we report for the first time the presence of filamentous fungi in Simuliidae larvae in Amazonia. Most of the fungal isolates used in this study have demonstrated during the in vitro bioassays with A. aegypti eggs, biotechnological potential for the control of this culicidian (Alencar et al. 2017). These discoveries and processes have all been made possible by advances in technology that allow for greater understanding and manipulation of fungal genomes and growth conditions. Fungal applications have already shown great promise in terms of their ability to improve efficiency and productivity in various industrial settings. As research continues to progress in this area, it is likely that even more exciting and impact applications will be discovered. The application of knowledge derived from research on fungi, is poised to be a particularly fruitful arena, with potential applications ranging from the development of new drugs to improvement in agricultural productivity (Roth et al.2023).

This study benefits humanity as it provides important information about the biology and classical taxonomy of filamentous fungi, many of which are not yet known to inhabit the intestine of Simuliidae larvae and which arouse interest in the development of biotechnological studies.

The objective of this study was to isolate and identify fungal species found on larvae of Simuliidae, collected directly in the field, helping to construct a data base that can be used in the future to support biotechnological studies.

## 2 MATERIALS AND METHODS

This study was conducted from September to October 2003 and from May to June 2004 in different localities in the municipalities of Manaus and Presidente Figueiredo (Amazonas) and Porto Velho (Rondônia) (Table 1).

Approximately 30 larvae of Simuliidae were collected using forceps, placed in containers with sterile distilled water and stored under refrigeration before being dissected in the laboratory. In the laboratory, Simuliidae larvae were dissected in distilled water with forceps and needles. Morphological characters used for species characterization were the same as those used in conventional studies of black fly systematics (Coscarón 1990, Hamada & Adler 2001). Voucher specimens are preserved in alcohol and deposited in the Invertebrate Collection of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Amazonas, Brazil.

Seven species of Simuliidae larvae were analyzed: Simulium perflavum Roubaud, Simulium. trombetense Hamada, Py-Daniel & Adler, Simulium maroniense Floch & Abonnenc, Simulium cauchense Floch & Abonnenc, Simulium daltanhani Hamada & Adler, Simulium rorotaense Floch & Abonnenc and Lutzimulium simplicicolor (Lutz). These insects were separated and stored in test tubes in groups of ten specimens and initially washed to remove saprophytic

microorganisms for 2 seconds in 70% alcohol and immersed in a solution of 4% sodium hypochlorite for 3 min and twice in sterile distilled water for 1 min each (Alves 1998). Each Simuliidae species pool was composed of 30 larvae that were macerated in 0.2 ml of Saline Solution (0.9%) in a Pachane Class II vertical laminar flow aseptic chamber.

The macerated samples were processed according to the technique of Alves (1998), 0.1 ml of this macerated was seeded on Petri dishes (9 cm) containing the following culture media Potato Dextrose Agar (DIFCO) to which 0.05 g per l of chloramphenicol were added. The plates were incubated at 28ºC and examined every three days for 20 days. The culture and identified colonies were transferred to test tubes (16 x 100 mm) containing 10 ml of PDA. These tubes were kept in a chamber under the same conditions of temperature (28ºC) and relative humidity (80%).

In order to observe the macroscopic characteristics of the fungi to allow identification of the genus of each isolate, fragments of the colony grown in the test tubes were transplanted using a platinum loop to Petri dishes containing the media Potato Dextrose Agar (PDA), Czapek-Dox-agar (CZ) and Malt Extract Agar (MEA) (DIFCO), incubated at 28ºC.

The cultures were identified by micromorphological characteristics for species identification, according to the method of Rivalier & Seydel (1932) and specific literature (Raper & Fennel 1965, Ellis 1971, 1976, De Hoog 1972, Hawksworth, 1977, Pitt 1979, 1985, Arx 1981, Klich & Pitt, 1994, Hawksworth et al. 1995, Klich, 2002). Species were mounted in Amann lactophenol plus cotton blue and observed under a compound microscope.

Voucher cultures were preserved in hemolysis tubes (15 × 100 mm) with PDA under a 1cm layer of mineral oil. The voucher cultures were incorporated into the Fungi Culture Collection of the Instituto Nacional de Pesquisas da Amazônia (INPA) and Coleção de culturas de Fungos do Departamento de Micologia, Instituto Oswaldo Cruz- FIOCRUZ/IOC.

Table 1. Simuliidae (Diptera: Nematocera) collection sites in Amazonia

| Site | Habitat | Date | Colletor | Longitude/Latitude |
|---|---|---|---|---|
| 1 | Road to Presidente Figueiredo County Cemetery | 18/05/2004 | Alencar, Y.B | 02º2'S, 60º39'W |
| 2 | HW AM010 km 24, Igarapé Acará, Reserva Florestal Adolpho Ducke, Manaus municipality, AM. | 07/10/2003 | Alencar, Y.B | 02º57'S, 59º57'W |
| 3 | HW AM240 km 20, Pousada Sossego da Pantera, Igarapé da Onça, Presidente Figueiredo municipality, AM. | 10/10/2003 | Alencar, Y.B | 02º02'S, 59º50'W |
| 4 | HW BR174 km 134, Road to Comunidade Castanhal, Igarapé Canoas, Presidente Figueiredo municipality, AM. | 10/10/2003 | Alencar, Y.B | 01º46'S, 60º28'W |
| 5 | HW AM010 km 51, km 4 of ramal CIGS (Candiru stream tributary), Manaus municipality, AM. | 22/10/2003 | Alencar, Y.B | 02º45'S, 59º51'W |
| 6 | Rio Bonito, Porto Velho municipality, Rondônia | 05/06/2004 | Ferreira, R.L.M., Silva, J.O., Hamada, N. | 02º59'S, 60º65'W |

Source: prepared by the authors (2023), HW: highway, BR: Federal highway, AM: Amazonas state highway

## 3 RESULTS AND DISCUSSION

The total of 87 fungal lineages were identified from homogenates of the larvae of seven black-fly species. Of the total fungi isolated, 22.9% were not identified to the species level due to the fact that some lineages remained sterile, even though a variety of larval medium and crop conditions were used to induce sporulation.

The present study indicates that Simuliidae larvae are a good component of aquatic systems for a number of filamentous fungi, ingested many material particulate in suspension. The micobiota were common on the larval homogenates in all seven black-fly species examined. Fifteen species of fungi were identified as: Cladosporium chlorocephalum (Fresen.), Cladosporium. herbarum (Pers.) Link, Penicilium crustosum Thom, Penicilium chrysogenum Thom, Penicilium lividum Westling, Penicilium citrinum Thom, Penicilium citreonigrum Dierckx, Penicilium corylophilum Dierckx, Penicilium fellutanum Biourge, Penicilium oxalicum Currie and Thom, Gliocladium virens Corda, Aspergillus japonicus Saito, Trichoderma harzianum Rifai, Trichoderma. koningii Oud. Aggr. and Pestalotiopsis guepini (Desmazieres) Steyaert. Table 2 shows the frequency of these fungi in the larvae of different species of Simuliidae. Larvae of S. perflavum had the greatest prevalence of anamorphic fungi when compared with other species of Simuliidae, with eight fungal species: P. chrysogenum, P. crustosum, P. lividum, P. corylophilum, T. harzianum, C. chlorocephalum, A. japonicus and P. guepinii.

P. guepinii was the most prevalent Simuliid collected, followed by P. fellutanum, T. harzianum and A. japonicus (Table 2).

Our results indicate the presence of several genera of entomopathogenic fungi and of fungi with industrial interest. Six genera were identified: Trichoderma, Penicilium, Aspergillus, Cladosporium, Pestalotiopsis and Gliocladium.

There are many studies of entomopathogenic fungal species that cause impact on host populations, but information on their biology, identification and the host-pathogen relationships is scarce. Entomopathogenic fungi are relatively common and they are the main pathogens of plant sucking insects (Wraight 1998, Arantes & Correia 1999).

The genera Trichoderma, Aspergillus and Penicillium have great biotechnological potential, and they, have been widely studied because of their metabolites of industrial interest. Production of the metabolite Trichoharzin from Trichoderma harzianum lineages, isolated from marine sponges has been reported (Osterhage et al. 2000).

Further studies in this area need to be undertaken with the objectives of selecting industrially important biocontrol agents. Entomopathogenic fungal species are cited as most effective in insect control programs (Hall & Papierok 1982, Messias 1989, McCreadie & Adler 2005).

Various researchers have isolated fungal species from insects. Fungal species found in natural association with adults of Musca domestica L. have been identified (Sales et al. 2002). The isolated fungi were identified as: Aspergillus flavus Link (23.8%), Aspergillus. niger Tiegh (14.4%), Penicillium corylophilum (21.4%), P. fellutanum (11.9%), Cladosporium cladosporoides (Fresen) de Vries (4.7%), Fusarium sp. (4.7%), Alternaria alternate (Fr.) Keissler (11.9%), Curvularia brachyspora Boedjin (2.4%), Mycelia sterilia (2.4%) and unidentified species in the order Mucorales (2.4%). Fungal species from Triatomidae have been isolated in Rio de Janeiro state (Moraes et al. 1998). Few workers have directly examined the endoperitrophic and endocuticular surfaces of the digestive tract of simuliid larvae and thus there is little available documentation of the microbial communities associated with these surfaces (Taylor et al. 1995). These authors reported that the structure and microbial flora of the digestive tract of larval Simulium ornatum Meigen from Canada were frequently devoid of bacteria, although the fungus Harpella melusinae Leger and Duboscq (Hapellales, Trichomycetes) was commonly attached to the endoperitrophic surface. In contrast, the endocuticular surface was regularly colonized by a diverse microflora composed of rod-shaped, coccoud, spiral, and filamentous bacteria and two species of Harpelalles (Trichomycetes).

Spore dispersal by insect vectors is recognized in many groups of fungi, including ascomycetes, basidiomycetes, imperfect fungi and zygomycetes (Ingold 1953, Kendrick 1985). Adaptations in various fungal groups appear to be the result of selection for arthropod dispersal. Many spores of basidiomycetes adhere to the legs and bodies of the flies, and insects may remove the entire slime layer, filled with basidiospores, within a few hours (Abbott 2002).

Knowledge of black-fly interactions with fungi can provide important information on larval nutrition and help to clarify differences observed in population productivity of larvae in different habitats in Amazonia. It was verified that microorganisms isolated from Amazonian dipterans have biotechnological characteristics, and when they are applied in researches targeting the isolation and increase of virulence of these microorganisms for controlling vectors, as well as the clarification of the mechanisms involved in the transmission of mycosis to the offspring, they become an expectation for the control of one of the major urban plagues that affects the public health and that worries the health authorities, the dengue fever transmission and others diseases.

In the actually, fungal lineages can be used for biological control of insect vectors of tropical diseases based on the selection of lineages that are potential producers of substances with economic demand in the region. The application of entomopathogenic fungal isolates that could infect one or more life stages of A. aegypti would be of great interest in integrated control efforts against this vector (Scholte et al., 2007). Entomopathogenic fungi had demonstrated potential for controlling mosquitoes in studies with B. bassiana and M. anisopliae over Anopheles gambiae, indicating a

possible reduction in malaria vector transmission in over 80% (Scholte et al., 2004, 2005; Blanford et al., 2005).

The study by Alencar et al. (2017) reported the discovery of the production process of an aqueous formulation that uses as a base filamentous fungi, endosymbionts, with an entomopathogenic character isolated from the intestine of immature Simuliidae and Amazonian plants, collected in ecosystems in the Brazilian Amazon region as ovicide and larvicide in the control of Ae. aegypti. This demonstrates the applicability and importance of new related research using fungal species for the biological control of insect vectors and agricultural pests.

Some limitations during the research were overcome through the identification of the fungi by Dr. Maria Inez de Moura Sarquis from the IOC/Fiocruz, Rio de Janeiro, who kindly collaborated to identify the lineages using classical taxonomy.

## 4 CONCLUSION

The research provided for the establishment of the collection of endosymbiont fungi of Insecta (Diptera) from the Amazon, currently stored at INPA. Knowledge about these fungi and their relationship to Simuliidae larvae can contribute to their use in biological control of insect vectors and also in biotechnological studies, with the selection of strains with economic potential. The importance of this study lies in the possibility of discovering new mycoinsecticides to control eggs and larvae of insect vectors of diseases in humans and other animals.

## ACKNOWLEDGMENTS

Financial and parcial support: FAPEAM/CNPq (Proc. PIPT/827/03, DCR/954/03) and INPA/CPEN. We thank M.I.M. Sarquis in memorian for taxonomic assistance and Philip Fearnside for reviewing the manuscript.

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Table 2. Fungal isolates obtained from Simuliidae larvae (Diptera: Nematocera) collected in different localities at Amazonia

| Species | *S. perflavum* [1](30) | *S. rorotaense* (30) | *S. trombetense* (30) | *S. cauchense* (30) | *S. maroniense* (30) | *S. daltanhani* (30) | *L. simplicicolor* (30) | Total (%) (210) |
|---|---|---|---|---|---|---|---|---|
| *P. chrysogenum* | 3 | - | - | - | - | - | - | 3 (3.4%) |
| *P. crustosum* | 1 | - | - | - | - | - | 1 | 2 (2.2%) |
| *P. lividum* | 2 | 1 | - | - | - | - | - | 3 (3.4%) |
| *P. fellutanum* | - | 7 | - | 2 | 1 | - | - | 10 (11.4%) |
| *P. citrinum* | - | 2 | - | - | - | - | 3 | 5 (5.7%) |
| *P. oxalicum* | - | 1 | - | - | - | 1 | - | 2 (2.2%) |
| *P. citreonigrum* | - | - | - | - | - | - | 1 | 1 (1.1%) |
| *P. corylophilum* | 1 | - | - | - | - | - | - | 1 (1.1%) |
| *T. koningii* | - | - | 3 | 1 | - | - | - | 4 (4.5%) |
| *T. harzianum* | 2 | 2 | 3 | 2 | - | - | - | 9 (10.4%) |
| *C. herbarum* | - | 1 | - | - | - | - | - | 1 (1.1%) |
| *C.chlorocephalum* | 1 | - | - | - | - | - | - | 1 (1.1%) |
| *G. virens* | - | 1 | - | 1 | 1 | 2 | - | 5 (5.7%) |
| *A. japonicus* | 2 | - | - | 3 | - | 3 | 1 | 9 (10.3%) |
| *P. guepinii* | 5 | 3 | - | 1 | 1 | 1 | - | 11 (12.6%) |
| *Mycelia sterilia* | 10 | 6 | - | - | - | 1 | 3 | 20 (22.9%) |
| Total | | | | | | | | 87 |

Source: prepared by the authors (2023); [1]number of dissected larvae, (-), absence.