

Identification, Conservation and Formation of a Bank of Strains of Quarantine Fungi Isolated from Bean Seeds and Tropical Forages, from the Germplasm Bank “Future Seeds”.

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Abstract

Reference strains are an important resource that makes it possible to guarantee the quality of test results, assess the quality of culture media and validate the methods used, mainly in phytosanitary diagnostic laboratories. This type of biological material is difficult to obtain, and its cross-border movement is also highly restricted, due to the risks of introducing pathogens to disease-free geographic areas. The Germplasm Health Unit (GHU) of the Bioversity International Alliance and CIAT is responsible for evaluating the presence of quarantine-type fungi in the bean and tropical forage collections of the “Semillas del Futuro” Germplasm Bank, which is why which is a priority for the GHU, to have this type of reference material available in order to carry out internal quality controls in accordance with the ISO 17025 standard. The GHU has create a bank of strains of quarantine fungi isolated from Seeds from the evaluations of the collections, as well as plant material with the presence of symptoms collected from the phytosanitary monitoring of the regeneration fields. In order to taxonomically characterize the strains obtained up to the species level, a DNA extraction protocol was developed and by amplifying the ITS (Internal Transcribed Spacer) region, several isolates were sequenced using Oxford Nanopore sequencing technology. The region sequenced will allow the implementation of complementary molecular tests (PCR and/or qPCR) in the routine diagnosis of the collections, strengthening the evaluation of quarantine-type fungi through phenotypic and genotypic characterization, guaranteeing the quality of the results obtained, which translates into an increase in the availability of the materials evaluated.

Keywords: Reference strain; Quarantine; ITS; Sequencing.

Introduction

The Genetic Resources Program (GRP) of the Alliance Bioversity International and CIAT, conserves the largest collections of beans (37,934 materials of *Phaseolus* spp.) and tropical forages (22,661 materials of 696 species). It is essential for the GRP to produce and preserve germplasm that meets the quality requirements for national and international distribution, including phytosanitary quality, which certifies all accessions distributed are free of quarantine pathogens. In beans and tropical forages seeds, are evaluated for the diagnosis of fungi, bacteria, and viruses. Quarantine fungi are one of the main agents that cause diseases in crops and significant economic losses in the agricultural sector (Doehlemann et al., 2017; Hariharan & Prasannath, 2021). For this reason, isolated strains are used as an important resource to guarantee the quality of the results, culture media and standardization of methodologies, mainly in phytosanitary diagnostic laboratories. Characterization of the fungal microbiome with massive sequencing can be performed by (i) non-targeted metagenomics, which seeks the total sequence of the DNA sample, or by (ii) amplicon-directed sequencing, which aims to sequence PCR products from barcode regions. Oxford Nanopore technology (ONT) sequencing not only allows complete sequencing of the ITS region (c.a. 500-800 bp), furthermore, offers a cost-effective approach for real-time massive sequencing assays in diagnostic laboratories (Gu et al., 2021).



Figure 1. The “Future Seeds” the Alliance of Bioversity International and the International Center for Tropical Agriculture (CIAT) genebank (Palmira, Colombia).

Methodology

1. Obtaining fungal colonies in pure cultures.

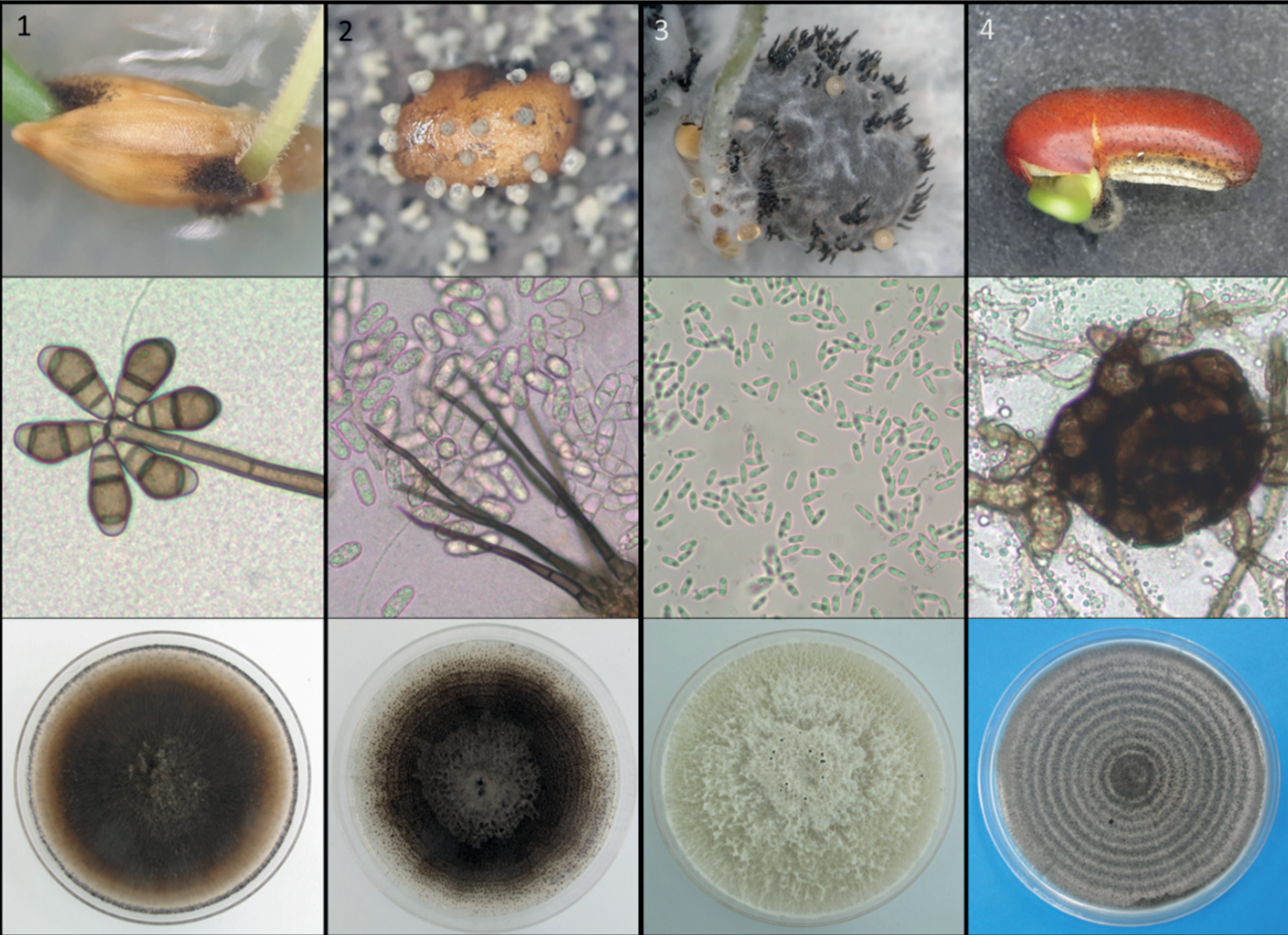
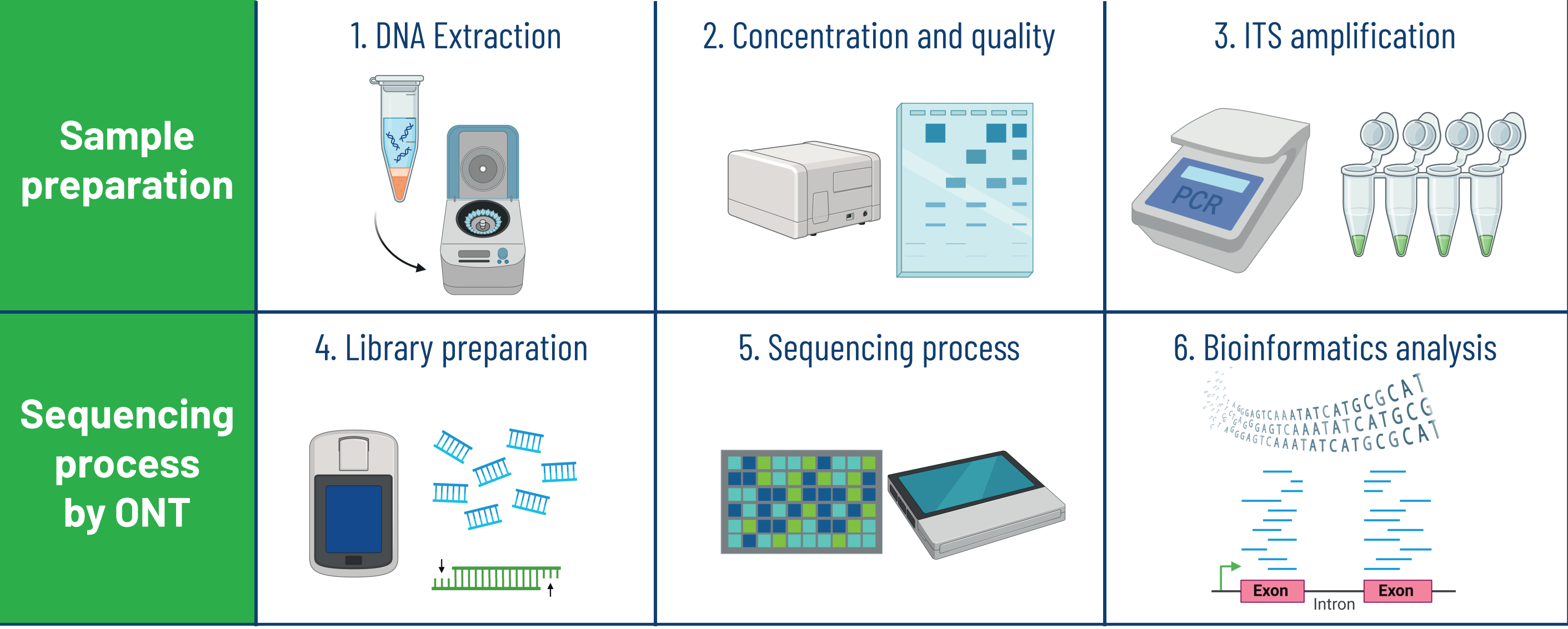


Figure 2. The fungal colonies obtained were isolated from the diagnostic process of samples collected in the phytosanitary monitoring in regeneration fields and seeds samples evaluated in the GHU laboratory.
(1) *Curvularia* sp.; (2) *Colletotrichum gloeosporioides*; (3) *Phomopsis* sp.; (4) *Macrophomina phaseolina*.

1. Extraction protocol, PCR, library, and sequencing process.



Results

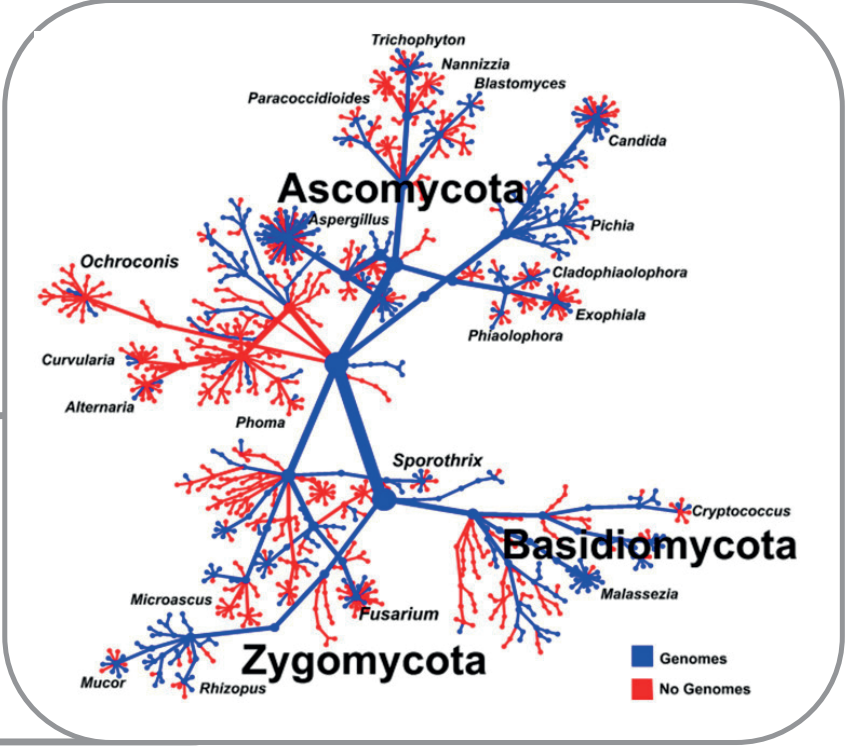
The sequencing library produced 0.69 M reads and 0.4 Gb that passed the kit's quality control (Phred score>8, length>200 bp), with a mean length of 562 (N50=569) and a mean Phred score of 11,797. The Kraken2 PlusPFP database (the most complete to date of the study) does not have genomic information associated with the genera *Phoma* (8), *Alternaria* (5), *Ascochyta* (1), or *Sclerotium* (1); therefore, 75% of the processed samples could not be confirmed at the genus or species level the morphological identification previously made. In this group, the average of unclassified readings was 68.63%. Of the 5 remaining samples, 3 (60%) coincided with the genus identified by morphology: *Colletotrichum* (11 and 17) and *Fusarium* (14) (Table 1). The samples morphologically identified as *Rhizoctonia solani* (WB14) (13) and *Colletotrichum* sp. CR-01 (20) were classified as *Fusarium*; however, this may be attributed to low sequencing depth and/or a lower percentage of reads classified as fungi.

Table 1. Taxonomic identification by ITS of fungal colonies. Samples are ordered from lowest to highest percentage ranked fungal reads. The genera that are represented in the database are shaded in yellow. In the intensity scale, the total number of readings per sample (green) and the percentage of unclassified readings (orange) are shaded.

Sample	Morphological identification	Identification by sequencing	Database representation	Total	Classified	Fungi	Fungi (%)	Not classified	Not classified (%)
1	<i>Phoma</i> sp.	<i>Colletotrichum higginsianum</i>	No	68,304	64,576	3,767	5.83	3,728	5.46
12	<i>Ascochyta</i> (Asc1)	<i>Colletotrichum higginsianum</i>	No	15,275	11,231	725	6.46	4,044	26.47
9	<i>Phoma</i> sp.	<i>Colletotrichum higginsianum</i>	No	9,159	4,002	399	9.97	5,157	56.31
13	<i>Rhizoctonia solani</i> (WB14)	<i>Fusarium oxysporum</i>	Yes	67	31	4	12.90	36	53.73
4	<i>Alternaria</i> sp. CR-033	<i>Ustilagoideae virens</i>	No	6,924	1,843	242	13.13	5,081	73.38
5	<i>Phoma</i> sp.	<i>Colletotrichum higginsianum</i>	No	7,168	1,833	260	14.18	5,335	74.43
7	<i>Alternaria</i> sp. CR-040	<i>Colletotrichum higginsianum</i>	No	88,382	87,333	16,207	18.56	1,049	1.19
15	<i>Sclerotium rolfsii</i> (Sr3)	<i>Marasmius oreades</i>	No	210	47	9	19.15	163	77.62
17	<i>Colletotrichum gloeosporioides</i>	<i>Colletotrichum higginsianum</i> *	Yes	267,291	248,783	52,997	21.30	18,508	6.92
3	<i>Alternaria</i> sp. CR-045	<i>Cercospora beticola</i>	No	1,085	157	34	21.66	928	85.53
2	<i>Alternaria</i> sp. CR-066	<i>Cercospora beticola</i>	No	4,072	754	177	23.47	3,318	81.48
16	<i>Phoma</i> sp.	<i>Cercospora beticola</i>	No	3,531	323	100	30.96	3,208	90.85
19	<i>Phoma</i> sp.	<i>Cercospora beticola</i>	No	13,043	898	323	35.97	12,145	93.12
8	<i>Phoma</i> sp.	<i>Cercospora beticola</i>	No	7,844	694	253	36.46	7,150	91.15
6	<i>Alternaria</i> sp. CR-066	<i>Cercospora beticola</i>	No	6,891	718	269	37.47	6,173	89.58
10	<i>Phoma</i> sp.	<i>Colletotrichum lupini</i>	No	18,352	1,623	650	40.05	16,729	91.16
18	<i>Phoma</i> sp.	<i>Cercospora beticola</i>	No	17,372	1,455	583	40.07	15,917	91.62
20	<i>Colletotrichum</i> sp. CR-01	<i>Fusarium</i>	Yes	656	58	24	41.38	598	91.16
11	<i>Colletotrichum gloeosporioides</i> (C.gloe1)	<i>Colletotrichum higginsianum</i> *	Yes	266	65	35	53.85	201	75.56
14	<i>Fusarium</i> sp. (F29)	<i>Fusarium falciforme</i> *	Yes	2,552	2,155	1,860	86.31	397	15.56

The low representation of genera such as *Phoma* and *Alternaria* had already been described by Hoang et al. (2022) (Figure 1). This limitation in the reference databases demonstrates the need to sequence these and other poorly characterized genera at the genomic level.

Figure 3. Tree of the fungal kingdom showing the main classes of fungi and their pathogenic species for humans, indicating species with (blue) and without (red) genomes. (Hoang et al., 2022).



Conclusions

The low representation of reference sequences in genera of quarantine interest shows the need to continue with the characterization by massive sequencing of these isolates obtained in the GHU. The greater number and percentage of readings belonging to fungi per sample, the probability of identifying the isolate at the species level increases. Despite the low availability of references in the databases, the Oxford Nanopore technology is presented as a promising technique for the verification at the species level of fungal colonies from the ITS region of isolates obtained from evaluations carried out in beans and tropical forages from the bank, which will allow the construction of a bank of reference strains for fungal diagnostic processes as positive controls and sequences to promote the development of molecular methodology in the GHU.

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