Detection of *Diaporthe* sp. in cacao plants (cv. CCN 51) in Guayas Province, Ecuador

Detección de *Diaporthe* sp. en plantas de cacao (cv. CCN 51) en la Provincia de Guayas, Ecuador

Detecção de *Diaporthe* sp. em plantas de cacau (cv. CCN 51) na província de Guayas, Equador

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Abstract

Cacao cultivation contributes significantly to the global economy. However, a decline in production is evident due to the presence of pathogens, especially within the fungi kingdom, where some remain unidentified. The objective of this study was to identify the presence of *Diaporthe* sp. in cacao plants of the CCN-51 cultivar in Ecuador. Samples of cacao branches with symptoms of rot and necrotic tissue were collected (cankers). The samples were disinfected and processed in the Microbiology Laboratory of the Milagro State University, Ecuador. Cambium fragments were cultured on potato dextrose agar (PDA) and incubated at 27 °C. After culture purification, morphological characterization and molecular identification were performed using ITS and EF1-α barcoding methods. The sequences were compared with the NCBI GenBank database for validation. A phylogenetic analysis was performed between the strains found and those reported in Puerto Rico and Australia. Morphological identification placed the isolates within the genus *Diaporthe*, which was confirmed molecularly. Phylogenetic analysis demonstrates marked genetic diversity among isolates within the genus *Diaporthe*. These findings suggest that *Diaporthe* spp. is prevalent in Ecuadorian cacao plantations and that molecular methods are effective for its identification. The presence of this pathogen implies the need for management strategies to mitigate its impact on cacao production.

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Resumen

El cultivo de cacao contribuye significativamente a la economía mundial. Sin embargo, es evidente la disminución de su producción a consecuencia de la presencia de agentes patógenos, en especial dentro del reino fungi donde algunos de ellos no se encuentran identificados. El objetivo del estudio fue identificar la presencia de *Diaporthe* sp. en plantas de cacao del cultivar CCN-51 en Ecuador. Se recolectaron muestras de ramas de cacao con síntomas de pudrición y tejido necrótico (cancros). Las muestras fueron desinfectadas y procesadas en el laboratorio de Microbiología de la Universidad Estatal de Milagro, Ecuador. Fragmentos del cámbium se cultivaron en agar papa dextrosa (PDA) e incubaron a 27 °C. Tras la purificación de cultivos, se realizó una caracterización morfológica y una identificación molecular mediante métodos de barcoding ITS y EF1-α. Las secuencias se compararon con la base de datos del NCBI GenBank para la validación. Se efectuó un análisis filogenético entre las cepas encontradas y las reportadas en Puerto Rico y Australia. La identificación morfológica situó a los aislamientos dentro del género *Diaporthe*, lo que fue confirmado molecularmente. El análisis filogenético demostró una marcada diversidad genética entre aislados del género *Diaporthe*. Estos hallazgos sugieren que *Diaporthe* spp. es prevalente en plantaciones de cacao en Ecuador y que los métodos moleculares son eficaces para su identificación. La presencia de este patógeno implica la necesidad de estrategias de manejo para mitigar su impacto en la producción de cacao.

Palabras clave: barcoding, cacao CCN-51, EF1-α, fitopatología, ITS.

Resumo

O cultivo do cacau contribui significativamente para a economia global. No entanto, um declínio na produção é evidente devido à presença de patógenos, especialmente dentro do reino dos fungos, onde alguns permanecem não identificados. O objetivo deste estudo foi identificar a presença de *Diaporthe* sp. em plantas de cacau da cultivar CCN-51 no Equador. Amostras de ramos de cacau com sintomas de podridão e tecido necrótico foram coletadas (cancros). As amostras foram desinfetadas e processadas no Laboratório de Microbiologia da Universidade Estadual de Milagro, Equador. Fragmentos de câmbio foram cultivados em ágar batata dextrose (PDA) e incubados a 27 °C. Após a purificação da cultura, a caracterização morfológica e a identificação molecular foram realizadas usando os métodos de código de barras ITS e EF1-α. As sequências foram comparadas com o banco de dados GenBank do NCBI para validação. Uma análise filogenética foi realizada entre as cepas encontradas e aquelas relatadas em Porto Rico e Austrália. A identificação morfológica situou os isolados dentro do gênero *Diaporthe*, o que foi confirmado molecularmente. A análise filogenética demonstra acentuada diversidade genética com alta similaridade dentro do gênero *Diaporthe*. Esses achados sugerem que *Diaporthe* spp. é prevalente nas plantações de cacau equatorianas e que métodos moleculares são eficazes para sua identificação. A presença desse patógeno implica a necessidade de estratégias de manejo para mitigar seu impacto na produção de cacau.

Palavras-chave: código de barras, cacau CCN-51, EF1-α, fitopatologia, ITS.

Introduction

Cocoa (*Theobroma cacao* L.), native to tropical America, has played an essential role in various cultures throughout history, prized for its many culinary, medicinal, and economic uses (Vera-Rodríguez *et al*., 2021). Ecuador, in particular, has experienced remarkable growth in its cocoa production. By 2019, 25,435 hectares of crops were registered, with an annual production of 283,680 tonnes, positioning the country as the third largest producer in the world (Vera *et al*., 2021). This increase is partly due to the decline in production in the main African producing countries, such as Côte d'Ivoire and Ghana, which has made Ecuador a preferred alternative for the global market (Colombatti Moran *et al*., 2024). The renowned quality and fine aroma of its cocoa, together with a solid international reputation, give Ecuador a significant comparative advantage over other markets (Córdova Durán, 2025).

Despite this, the incidence of disease in cocoa crops is a growing concern globally, posing a significant threat to the economy and agricultural sustainability (de Novais *et al*., 2023; Cobos Mora *et al*., 2024). Fungal diseases can devastate entire crops, drastically reducing production and affecting cocoa quality, with direct repercussions on the chocolate industry (Fernández Muñoz *et al*., 2022). Furthermore, the prevalence of these diseases not only compromises the economic viability of farmers, but also threatens the livelihoods of families who depend on cocoa as their main source of income (Ramón Guanuche *et al*., 2024). Recent research highlights the vulnerability of cocoa to a wide range of rapidly spreading diseases with devastating effects, underscoring the urgency of effective management measures (Rêgo *et al*., 2023).

The impact of fungi and oomycetes on cocoa cultivation is a significant challenge faced by cocoa producers worldwide (Perrine-Walker, 2020). Pathogens such as *Moniliophthora roreri*, *Moniliophthora perniciosa*, *Lasiodiplodia theobromae*, and *Phytophthora palmivora* cause devastating diseases such as moniliasis, witches' broom, pod rot, and plant rot, respectively, which can drastically reduce harvests in a matter of weeks (Delgado-Ospina *et al*., 2021; Chóez-Guaranda *et al*., 2023; Puig, 2023). These diseases deteriorate the quality and quantity of cocoa produced, negatively impacting the economy of producers and the chocolate industry (Rodríguez Velázquez *et al*., 2024).

The spread of fungal diseases in cocoa crops is intensified by factors such as climate change, deforestation, and the lack of sustainable agricultural practices (Delgado-Ospina *et al*., 2021). Often, the genetic resistance of cocoa varieties is insufficient to counteract the rapid evolution of pathogenic fungi (Ruiz-Chután *et al*., 2024). Therefore, addressing these challenges requires a comprehensive approach that includes the development of resistant varieties, the implementation of sustainable agricultural practices, and cooperation between producers, governments, and international organisations (Ramos *et al*., 2024; Sousa *et al*., 2024).

In recent years, new fungi have been identified that pose a significant threat to cocoa production, intensifying concern among producers, researchers, and the industry in general (Debnath *et al*., 2023; Chávez *et al*., 2024). The identification in Puerto Rico of *Diaporthe tulliensis* and *Diaporthe pseudomangiferae* as causal agents of pod rot in cocoa underscores the importance of recognising and understanding new pathogens. This finding is the first report of these species affecting cocoa, highlighting the urgent need to develop effective control measures (Serrato-Diaz *et al*., 2022).

The genus *Diaporthe* is now considered paraphyletic according to Gao *et al*. (2017), the asexual state formerly known as *Phomopsis* (Zhu *et al*., 2023), includes pathogenic fungi that affect a wide variety of plants, including agricultural crops, fruit trees, and ornamental plants, with a considerable impact on agricultural production and ecosystem health (Mena *et al*., 2023). Several species of *Diaporthe* cause symptoms such as leaf spots, wilting, root rot, and branch dieback, which can have devastating consequences for the yield and health of host plants (Sánchez *et al*., 2015). These fungi play a variety of ecological roles, acting as pathogens, endophytes, or saprophytes, which underscores the importance of studying and understanding them for effective management (Thompson *et al*., 2015; Liu *et al*., 2024).

Cocoa producers face the difficult task of protecting their crops from fungal diseases, unaware of the presence of new pathogenic fungi that threaten them, while seeking to maintain the profitability of their business. The study aimed to identify the presence of the fungus *Diaporthe* sp. in CCN-51 cocoa plants from crops in Ecuador.

Materials and methods

The research was carried out in the Hermanos Quito area (2°11'45.468" S, 79°18'36.486" W, 105 m.a.s.l), Lorenzo de Garaicoa Parish, Simón Bolívar Canton, northeast of the Province of Guayas, Ecuador, during the summer season (September, 2024). Three farms (A, B and C) with CCN-51 cocoa plantations showing symptoms of branch canker (Figure 1) were taken as a reference. The 10-year-old plants were planted in loamy soil at a planting distance of 3 x 3 m between plants and rows. The farms have a subfoliar irrigation system. According to reports from producers, the plantations have suffered a loss of fruiting of around 40 % caused by these symptoms in the plants. The affected plants show generalised necrosis on branches (Figure 1: A1, B1, C1), cankers on branches (Figure 1: A2, B2, C2) and rot at the base of the trunk (Figure 1: A3, B3, C3).

Four plants with these symptoms were randomly selected from each farm, and four samples of branches showing signs of necrosis were taken from each plant.

The samples were processed in the Microbiology Laboratory of the Biotechnology Department of the Faculty of Science and Engineering at the Universidad Estatal de Milagro (UNEMI) in Ecuador. Following the suggestions of Jiménez *et al*. (2022), the samples were washed with neutral soap and rinsed with distilled water, then disinfected by immersing them in 1 % sodium hypochlorite for 1 minute, followed by washing with distilled water. Next, with the aid of a scalpel, the branches were cut transversely into pieces of approximately 5 cm, placed in 70 % ethanol for 1 min and then in the safety cabinet (BIOBASE model FH1200(X), China) until processing. Small fragments near the necrotic tissue were then cut, the bark was removed, and a cut of approximately 1 cm² was made in the cambium. The plant tissue samples were individually seeded in the centre of Petri dishes with potato dextrose agar (TM MEDIA) in triplicate and incubated at 27 °C for eight days. The cultures were then purified by transplanting hyphae with the microbiological loop into four new plates with PDA (TM MEDIA) and incubated at 27 °C for 14 days.

Subsequently, a macroscopic morphological characterisation of the purified fungal cultures was carried out to identify characteristics such as shape, colour, and texture. Microscopic morphological characterisation was performed using lactophenol blue staining and observation of the samples under a trinocular microscope (40x objective, Motic™, model Panthera S., China). Given the marked uniformity in the macroscopic characteristics observed in the isolated fungal colonies, 25 % of the isolates from each farm were selected for molecular identification.

Molecular typing of the fungal isolates was performed using barcoding techniques based on ITS and EF1-α markers. DNA extraction was performed using the commercial Mini Genomic DNA FUNGUS kit (Biotium®), following the manufacturer's instructions,

Figure 1. A1, B1, C1) Generalised necrosis in branches; A2, B2, C2) Cankers in branches; A3, B3, C3) Rot at the base of the trunk.

from approximately 100 mg of mycelium scrapings from each fungal sample (Barh *et al*., 2023).

DNA integrity and quality were assessed using microvolume spectrophotometry with NanoDrop (Thermo Scientific ND 2000C, USA) and 1 % agarose gel electrophoresis. The DNA was then diluted to a concentration of 20 ng.µL^{-1} and used for polymerase chain reaction (PCR) amplification, using primers from Macrogen and BlasTaq™ ITS1/ITS4 and EF1-983F/EF1-2218R, respectively (Rehner and Buckley, 2005; White *et al*., 1990). The PCR conditions were as follows: initial denaturation (98 °C, 1 min), followed by 40 cycles of denaturation (94 °C, 30 s), annealing (60 °C for ITS and 65 °C for EF1-α, 15 s), and extension (72 °C, 1 min). Finally, the final extension (72 °C, 10 min) and maintenance at 8 °C were performed. The final reaction volume was (20 µL). Subsequently, the amplified products were sequenced in the 5' → 3' direction using the Sanger method, using the same primers used for the amplification of each gene.

The resulting sequences were cleaned and assembled using Geneious bioinformatics software version 11.1.2. Finally, the sequences assembled and aligned by BLAST were compared with the NCBI GenBank nucleotide database to determine the taxonomic identification of the isolates.

Partial sequences obtained from the ITS and EF1-α genes under study were used to design the phylogenetic tree, as well as strains referenced by Thompson *et al*. (2015) and Serrato-Diaz *et al*. (2022) deposited in GenBank, which were individually aligned using the MUSCLE algorithm with the MEGA X programme, ensuring accurate alignment. Subsequently, the aligned sequences of both regions were concatenated in FASTA format, joining the homologous sequences by strain and checking their correct correspondence by code.

Phylogenetic analysis was performed in this same software using the Maximum Likelihood method under the Tamura-Nei substitution model with 1000 bootstrap replicates, measuring evolutionary distances as the number of substitutions per site. Finally, the Tree Explorer tool in MEGA X was used to visualise and edit the tree, adjusting labels, topology and distances, highlighting strains of interest through their respective codes to facilitate their identification in the comparative analysis.

Results and discussion

Morphological characterisation

Macroscopic morphological characterisation of randomly selected colonies of the strains revealed irregular edges and a soft biomass with white cottony growth (Figure 2).

Figure 2. Macroscopic morphology of *Diaporthe* spp. isolates after 14 days of growth on PDA. H630, H767, H768: code of isolates corresponding to a different farm.

Conidiophores (small black pustules) were observed after 30 days, characteristic of the asexual reproductive phase of the fungus for spore release (Figure 3B). Findings consistent with morphological characteristics described by Santos *et al*. (2011) and Vidić *et al*. (2011).

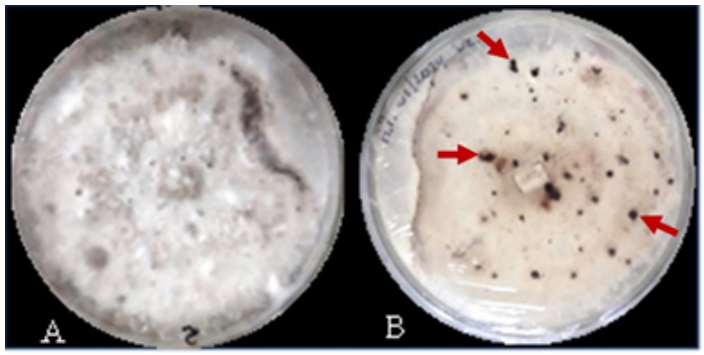

Figure 3. Macroscopic morphology of *Diaporthe* spp. isolation after 30 days of growth on PDA. View of the plate: A (top) growth of the fungal colony, B (bottom) formation of reproductive structures.

Figure 4 shows the microscopic structures of the fungus, highlighting the presence of mycelium with its septate and hyaline hyphae (Figure 4A). Its conidiophores (Figure 4B) and light-coloured cylindrical conidia (Figure 4C) can also be seen, with characteristics that confirm the genus *Diaporthe* sp., similar to the findings of Gao *et al*. (2017).

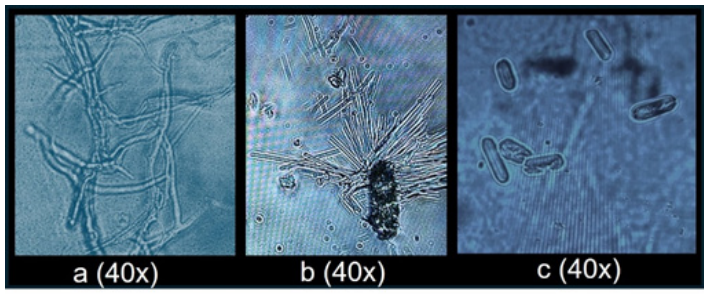

Figure 4. Microscopic morphology of the H630 *Diaporthe* spp. isolate after 30 days of growth. a (fungal mycelium and hyphae), b (conidiophores), c (conidiospores). Motic Images Plus 3.0 software. Images captured at 40x magnification.

In this context, recent research has delved deeper into the study of the *Diaporthe* genus. For example, Long *et al*. (2019) highlighted the use of morphological data and molecular data using key genes for the classification of species within this genus. Furthermore, Wang *et al*. (2021) reported the identification of 52 new species of *Diaporthe* that infect a variety of plants, acting as endophytes, saprophytes, or pathogens. This finding is supported by morphological, phylogenetic, and molecular evidence, as confirmed by Perera *et al*. (2018).

Molecular identification

For molecular characterisation, high-quality DNA (A260/A280 ~2.05) was extracted, allowing efficient amplification, with bands of approximately 650 bp for the ITS marker and 1,200 bp for the EF1-α marker (Figure 5).

Table 1 shows a summary of the results of the molecular identification of the fungal isolates. The sequences obtained from Sanger sequencing confirmed the presence of the following species in the samples with a high level of identity: H630, H767, and H768. Accession No. OP753556.1; OP698111.1 under fragment EF1-α (*D. longicolla*); H630 Accession No. NR_147535.1 for the ITS fragment (*D. miriciae*); H767 and H768 Accession No. MF070235.1 by means of the ITS gene (*D. ueckeri*).

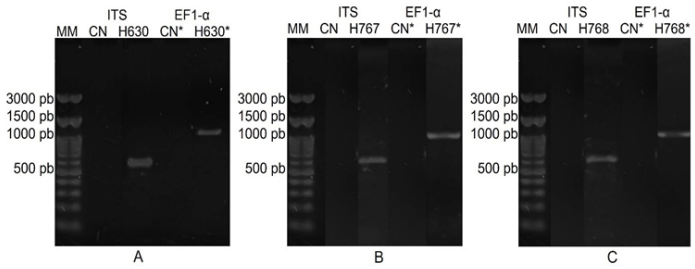

Figure 5. 1% agarose gel with PCR products for ITS and EF1-α fragments. MW: Molecular weight marker, NC: ITS negative control, NC*: EF1-α negative control. bp = base pairs. (A = H630; B = H767 and C = H768): Code for Diaporthe spp. isolates.

The identification of isolates as *D. longicolla* with the EF-α gene indicates that this fungus could be the causal agent of the symptoms observed in cocoa. Many fungi of the genus *Diaporthe* are known phytopathogens that cause rot in a wide range of hosts. In fact, recent studies by Sun *et al*. (2021) reported the first case in China of *D. longicolla* causing leaf spots on *Kalanchoe pinnata*, a medicinal plant. Meanwhile, Mena *et al*. (2024) reported the first case of *D. miriciae* as the cause of soybean stem canker in Uruguay. Dos Santos *et al*. (2024) presented the first report of soybean seed rot caused by *D. ueckeri* in Brazil, while Guo *et al*. (2024) reported the first case in China of brown spot caused by *D. ueckeri* in bottle palms (*Hyophorbe lagenicaulis*).

Phylogenetic analysis

The phylogenetic tree shown in Figure 6 shows the evolutionary relationships between different species of the genus *Diaporthe* reported (*) by Thompson *et al*. (2015) and Serrato-Diaz *et al*. (2022), and the sequences with which they showed the greatest identity when performing the Blast of the strains under study (OBJ), using concatenated data from the ITS and EF1-α regions, and rooted using the species *Diaporthella corylina*, reported in the study by Wang *et al*. (2021), as an outgroup.

The isolates from the study were grouped into three different species: two sequences as *D. miriciae* (OBJ1 and OBJ2), one as *D. ueckeri* (OBJ3) and another as *D. longicolla* (OBJ4). In all cases, the OBJ sequences were positioned within the clades corresponding to the reference species deposited in GenBank, with high support values (≥ 97 %), confirming their correct identification.

In particular, the *D. longicolla* clade showed a bootstrap support of 99 %, clearly grouping isolate OBJ4 with reference sequences KC343004.1 and OP753556.1. Similarly, isolate OBJ3 was grouped with *D. ueckeri* (MF070235.1) with 97 % support, while isolates OBJ1 and OBJ2 were grouped with *D. miriciae* (KJ197283.1 and NR_147535.1), also with 97 % support. These results demonstrate the consistency between the sequences obtained in this study and those reported, ensuring the reliability of species-level identification.

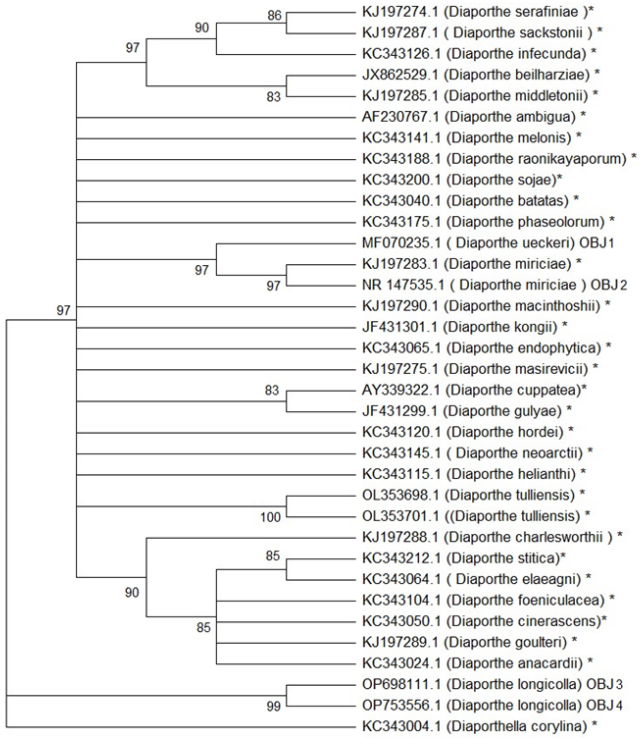

Figure 6. Phylogenetic tree based on partial sequences of the ITS and EF1-α genes with the highest identity when performing the Blast of the strains under study (OBJ) and strains reported (*) by Thompson *et al.* (2015), Serrato-Diaz *et al.* (2022) and Wang *et al.* (2021).

The identification of the genus *Diaporthe* as a pathogen allows for a better understanding of the aetiology of diseases that could previously be attributed to other factors or pathogen complexes (Mena *et al*., 2023). This knowledge is crucial for developing more effective and targeted management strategies, including the implementation of preventive measures, the development of resistant cultivars, and the timely application of specific phytosanitary treatments (Chávez *et al*., 2024). Diaporthe's ability to cause a variety of symptoms in different parts of the plant, such as cankers, leaf spots, and fruit rot (Patiño-Moscoso *et al*., 2023), underscores the need for rigorous phytosanitary surveillance to prevent significant economic losses in agriculture.

Table 1. Summary of molecular identification results for *Diaporthe* spp. isolates from CCN-51 cocoa plants showing disease symptoms in Hermanos Quito, Lorenzo de Garaicoa Parish, Simón Bolívar Canton, northeast of Guayas Province.

Code	DNA quality (%)	Organism	Fragment	Identity (%)	Nº Accession	Coverage (%)	E-value
H630	100	*Diaporthe longicolla*	EF1-α	98.41	OP753556.1	100	0.0
	100	*Diaporthe miriciae*	ITS	99.26	NR_147535.1	100	0.0
H767	89.5	*Diaporthe longicolla*	EF1-α	96.98	OP753556.1	100	0.0
	95.2	*Diaporthe ueckeri*	ITS	99.38	MF070235.1	100	0.0
H768	100	*Diaporthe longicolla*	EF1-α	98.45	OP698111.1	100	0.0
	100	*Diaporthe ueckeri*	ITS	99.82	MF070235.1	100	0.0

Conclusions

The macro and micro-morphological characteristics observed in the colonies allowed the fungal isolates to be identified within the genus *Diaporthe*, which was confirmed molecularly. Likewise, phylogenetic analysis of the strains found versus those reported in Australia and Puerto Rico shows marked genetic diversity; although they are grouped separately, they maintain a high similarity within the genus *Diaporthe*.

Further studies are recommended to explore the pathogenicity of these species and their interaction with different cocoa cultivars, facilitating early detection and rapid response to new outbreaks.

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