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Phylogenomics and gene selection in *Aspergillus welwitschiae*: Possible implications in the pathogenicity in *Agave sisalana*

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ABSTRACT

Aspergillus welwitschiae causes bole rot disease in sisal (*Agave sisalana* and related species) which affects the production of natural fibers in Brazil, the main worldwide producer of sisal fibers. This fungus is a saprotroph with a broad host range. Previous research established *A. welwitschiae* as the only causative agent of bole rot in the field, but little is known about the evolution of this species and its strains. In this work, we performed a comparative genomics analysis of 40 Aspergillus strains. We show the conflicting molecular identity of this species, with one sisal-infecting strain sharing its last common ancestor with *Aspergillus niger*, having diverged only 833 thousand years ago. Furthermore, our analysis of positive selection reveals sites under selection in genes coding for siderophore transporters, Sodium-calcium exchangers, and Phosphatidylethanolamine-binding proteins (PEBPs). Herein, we discuss the possible impacts of these gene functions on the pathogenicity in sisal.

1. Introduction

Aspergillus welwitschiae is a fungal species of the phylum Ascomycota [1], named after *Welwitschia mirabilis*, a peculiar plant species endemic to the Namib desert, where the fungus was first reported [2,3]. *A. welwitschiae* naturally inhabits the soil and spreads its spores in the air, and may cohabit with plants and cause primary pathology, followed by plant death [4,5]. *A. welwitschiae* is a saprotroph and also an opportunistic pathogen with necrotrophic behavior [6]. The current research discusses some aspects of *A. welwitschiae* in the context of the red rot (or bole rot) disease of *Agave sisalana*.

Agave sisalana and some varieties of the *Agave* genus, commonly known as "sisal" [7,8], are plants of economic interest associated with the natural fibers extracted from their leaves [9]. Brazil is the largest producer of these fibers, with the cultivation areas mostly concentrated in the semiarid region of Brazil, mainly in the state of Bahia, which

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accounts for 93% of national production and 40% of world production of sisal, and, to a lesser extent, in Paraíba and other states in the Northeastern region of Brazil [10,11]. The sisal culture is essential for the population living in this region, as it is one of the few and main sources of income [12]. The bole rot is one of the most relevant limiting factors for the cultivation of sisal in these regions and the consequent production of natural fibers, causing great production losses. This disease affects the plant stems and is characterized by an intense red color in affected stems and leaf basis, followed by chlorosis (yellowing of the leaves) and subsequently death [13].

The bole rot disease is caused by *A. welwitschiae*, which was assigned in a study published in 2018, after decades of isolated and incomplete studies of this pathosystem [13]. Before that study, the etiologic agent of the bole rot was thought to be *A. niger* while some other species were also assigned [14,15]. A possible explanation for this misclassification would be that both *A. welwitschiae* and *A. niger* belong to section Nigri of the genus *Aspergillus*, which is a non-formal name for a group of closely related species, undistinguishable by almost any morphological features, and forming a monophyletic clade [16]. Few studies focus on the evolution and phylogenomics of some sections of the genus *Aspergillus* [17,18]. None of these studies, however, perform a broad analysis of the genus, approach the evolution of pathogenicity in *A. welwitschiae,* or even include this species. There are currently five available assemblies of *Aspergillus welwitschiae*, for the following strains; CBS 139.54 [1], CCMB 674 [19], CCMB 663 (this study), ITEM 11945 [20], and IHEM 2864 (unpublished). Only the first three of them are annotated and were, thus, used in this study. Two of these genomes were obtained from *A. sisalana*, and the strain CCMB 663 is presented in this current study.

In this context, we present a comparative genomic study of several *Aspergillus* species, comprising species in the section Nigri of *Aspergillus*, focusing on the unique characteristics that evolved in *A. welwitschiae*, including a new strain from sisal. In addition, we aimed to analyze effector proteins (predicted by their corresponding enzyme-coding genes), hypothesized to play a key role in colonization and intracellular interaction between plant and fungus [19], culminating in the apparent and typical symptoms that give rise to the popular name of this plant disease. The broad spectrum of living and decomposing hosts and its consequent broad range of metabolic strategies summarizes the relevance of the genomics and evolution of *A. welwitschiae.*

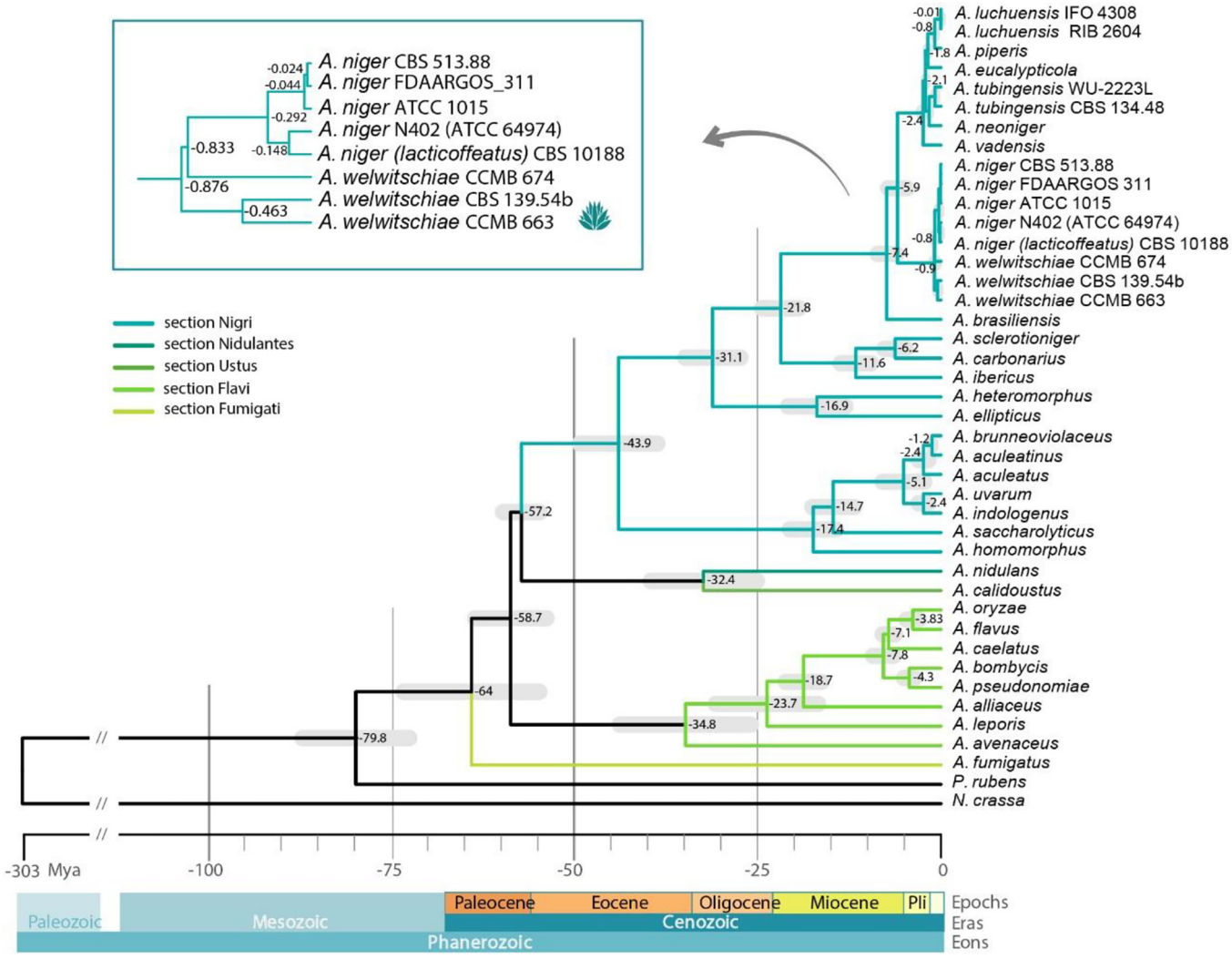

Fig. 1. Phylogenomic tree of *Aspergillus* species. Maximum likelihood reconstruction using 1842 single copy orthogroups alignment matrix. Branch support is 100% for all branches, obtained with 1000 bootstraps. Tree dating used the two calibration points of nodes in bold, and other node ages were estimated by the least-squares method in IQtree. Branches are colored by section as described in Vesth et al. (2018) [1]. *Neurospora crassa and Penicillium rubens* are used as outgroups. The *A. niger/ A. welwitschiae* clade is zoomed in the detail box showing the paraphyletic status and the node ages of *A. welwitschiae* lineages.

2. Results

2.1. Homology assignment and phylogenomic reconstruction

We used 42 fungal genomes for ortholog assignment; 40 *Aspergillus* genomes distributed among sections Nigri (21 species), Flavi (8 species), Nidulantes, Ustus, and Fumigati (one species each), plus 2 outgroups, *Penicillium rubens* and *Neurospora crassa*. Species in the section Nigri helped increase the resolution of our analysis, while further distant species in the genus *Aspergillus* and other species outside of this genus helped for a broader perspective of gene conservation (Table S1). For this dataset, we found 1842 families of single-copy ortholog (SCO) genes (Supplementary data). We used SCO to reconstruct the phylogeny of this species, obtaining 100% of bootstrap support for all branches (Fig. 1). The three *A. welwitschiae* genomes are positioned in the section Nigri, in the same clade as *A. niger*, which was expected since *A. welwitschiae* is a morphological cryptic species of *A. niger*. Nonetheless, interestingly, the three *A. welwitschiae* strains are polyphyletic: *A. welwitschiae* CCMB 663 and *A. welwitschiae* CBS 139.54 are in a monophyletic clade whereas *A. welwitschiae* CCMB 674 is clustered with the other *A. niger* species (Fig. 1, detail).

Time calibration estimated a divergence time of 463 thousand years (326–659C.I.) for the CCMB 663 and CBS 139.54 lineages of *A. welwitschiae*, which is longer than the divergence times among *A. niger* lineages. An even greater timespan was estimated for the MRCA of *A. welwitschiae* CCMB 674 and *A. niger*, dated 833 thousand years (609–1088C·I). The MRCA of the *A. niger/A. welwitschiae* clade dated 876 thousand years (609–1293C.I), which is much more recent than the ones separating other *Aspergillus* species, for instance, the MRCAs of *A. brunneoviolaceus/A. aculeatinus* (1.2Mya), and *A. sclerotionger/A. ibericus* (6.2Mya) (Fig. 1).

2.2. Prospecting for positive selection evidences in A. welwitschiae strains

As described by the developers of HyPhy [21], BUSTED [22] "provides a gene-wide (not site-specific) test for positive selection by asking whether a gene has experienced positive selection in at least one site on at least one branch", while MEME [23] "aims to detect sites evolving under positive selection under a proportion of branches". In our study, these two methods for inferring positive selection pressure are combined with the purpose of (i) detecting genes that are under positive selection in *A. welwitschiae* but not in any other species in our analysis, and consequently, (ii) differentiating the gene usage and presumably pathogenicity strategies in *A. welwitschiae* that differ from those other species. From 1842 SCO orthogroups tested using the BUSTED model, 64 presented evidence of positive selection when comparing the three *A. welwitschiae* strains to the other 39 fungal species [22]. These 64 orthogroups were also tested using the MEME branch-site model that confirmed positive selection evidence in the same 64 orthogroups. Among them, 28 families presented a p-value <0.0001 and were chosen for further annotation with InterPro and Pfam using GoFeat [24]. The consensus terms of InterPro and Pfam (meaning terms showed in all three strains) were obtained for the *A. welwitschiae* genes in 25 gene families, out of which 10 gene families have been previously described in the literature with roles in pathogenicity (Table 1). The other 15 gene families under positive selection have no specific effects on pathogenicity described in the literature (Table S2).

Guided by the strong literature support for the role of their detected domains in pathogenicity, we decided to further investigate two ortholog families under positive selection (OG3308 and OG4361), by checking sites under selection (detected with the MEME model). The OG3308, which contains a Major Facilitator Superfamily domain according to Interproscan and Pfam, was annotated as an iron transporter protein (Fig. 2A). In this gene family, two mutations were detected in *A. welwitschiae* strain CCMB 674 (ON695840) and one mutation in strain CBS 139.54 (RDH30067.1). In the latter, the glycine in site 738 is present in the other *A. welwitschiae* strains, and in an *A. niger* strain is exchanged for a serine, as so happens in other *A. niger* strains. In the MFS domain, which is also part of the siderophore domain, strain CCMB 674 and two other *A. niger* strains exchange the typical serine for alanine. Finally, in site 37 (cytoplasmatic domain/coil) strain CCMB 674 contains a valine instead of the alanine seen elsewhere. No mutations were

Table 1
Gene clusters with positive selection evidence and previously associated with pathogenicity, and their detected InterPro and Pfam codes (with respective descriptions) present in all three *A. welwitschiae* strains.

Cluster	Protein families and domains
OG3308	IPR020846: Major facilitator superfamily domain IPR036259: MFS transporter superfamily IPR011701: Major facilitator superfamily PF07690: *MFS_1* (Major Facilitator Superfamily)
References: Pel et al., 2007 [25]; Roohparvar et al., 2007 [26]; Wang et al., 2012 [27]; Menke et al., 2013 [28]; Natesan et al., 2013 [29]; Che et al., 2018 [30].	
OG3525	IPR003163: Transcription regulator HTH, APSES-type DNA-binding domain IPR018004: KilA, N-terminal/APSES-type HTH, DNA-binding IPR020683: Domain of unknown function DUF3447 IPR036770: Ankyrin repeat-containing domain superfamily IPR036887: HTH APSES-type DNA-binding domain superfamily
References: Donaldsson et al., 2013 [31]; Zhao et al., 2014 [32]; Rath et al., 2020 [33]; Xin et al., 2020 [34];	
OG3961	IPR027267: AH/BAR domain superfamily IPR028245: Eisosome component PIL1/LSP1 PF13805: *Pil1*(Eisosome component PIL1)
References: Suh et al., 2012 [35]; Athanasopoulos et al., 2013 [36]; Zhang et al., 2017 [37]; Colou et al., 2019 [38];	
OG4087	IPR002110: Ankyrin repeat IPR020683: Domain of unknown function DUF3447 IPR036770: Ankyrin repeat-containing domain superfamily IPR036887: HTH APSES-type DNA-binding domain superfamily IPR018004: KilA, N-terminal/APSES-type HTH, DNA-binding IPR029793: MBF transcription factor complex subunit Mbp1/Res1/Res2 IPR003163: Transcription regulator HTH, APSES-type DNA-binding domain PF00023: *Ank* (Ankyrin repeat) PF04383: *KilA-N* (KilA-N domain)
References: Medina et al., 2019 [39]; Hussein et al., 2011 [40].	
OG4150	IPR018327: Rad4 beta-hairpin domain 2 IPR004583: DNA repair protein Rad4 IPR038765: Papain-like cysteine peptidase superfamily IPR018325: Rad4/PNGase transglutaminase-like fold IPR018326: Rad4 beta-hairpin domain 1
References: Li et al., 2010 [41]; Feng et al., 2020 [42]; Paul et al., 2021 [43].	
OG4361	IPR004837: Sodium/calcium exchanger membrane region IPR005185: Inner membrane component domain PF01699: *Na_Ca_ex* (Sodium/calcium exchanger protein) PF03733: *YccF* (Inner membrane component domain)
References: Kmetzsch et al., 2010 [44]; Hu et al., 2014 [45]; Klukovich et al., 2016 [46]; Qi et al., 2022 [47].	
OG4394	IPR034753: hSac2 domain IPR022158: Inositol phosphatase IPR002013: SAC domain PF12456: *hSac2* (Inositol phosphatase) PF02383: *Syja_N* (*Sac*I homology domain)
References: Sheng et al., 2015 [48]; Zhang et al., 2015 [49].	
OG4836	IPR029058: Alpha/Beta hydrolase fold IPR002921: Fungal lipase-like domain PF01764: *Lipase_3* (Lipase (class 3))
References: Stehr et al., 2003 [50]; Brunke & Hube, 2006 [51]; Gupta et al., 2015 [52].	
OG4911	IPR000961: AGC-kinase, C-terminal IPR011009: Protein kinase-like domain superfamily IPR000719: Protein kinase domain PF00069: *Pkinase* (Protein kinase domain)
References: C. Wang et al., 2019 [53]; Y. Wang et al., 2019 [54]; Fabri et al., 2019 [55].	
OG4981	IPR008937: Ras-like guanine nucleotide exchange factor IPR000651: Ras-like guanine nucleotide exchange factor, N-terminal IPR019804: Ras guanine-nucleotide exchange factor, conserved site IPR023578: Ras guanine nucleotide exchange factor domain superfamily IPR001895: Ras guanine-nucleotide exchange factors catalytic domain
References: Gu et al., 2017 [56]; Martin-Vicente et al., 2019 [57].	

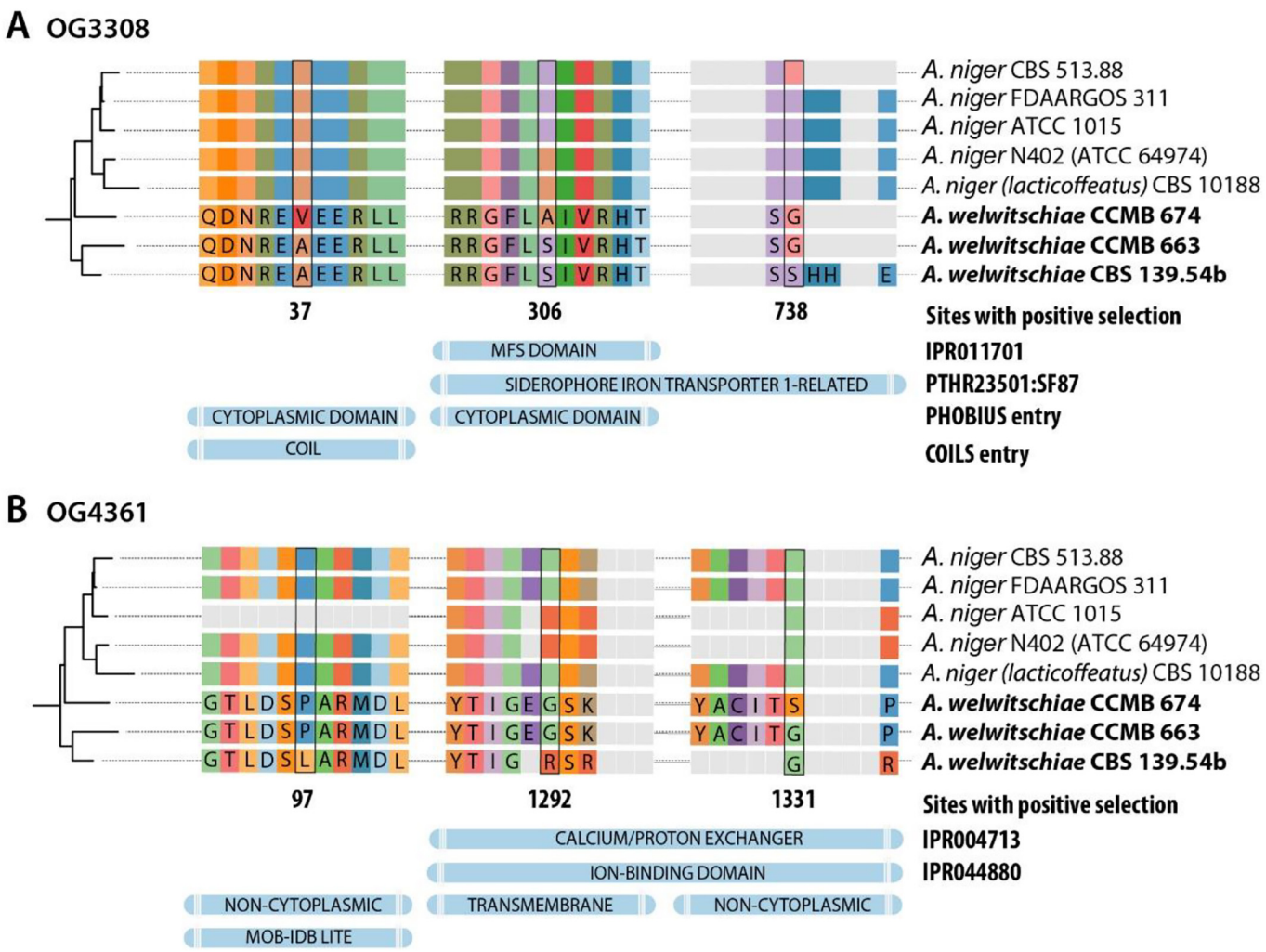

Fig. 2. Sites with positive selection for OG3308 (A), coding an iron transporter protein, and OG4361 (B), coding for a Na^{+}/Ca^{2+} exchanger. The figure shows a 10-amino acid region around three alignment sites with positive selection evidence ($p < 0.05$). The corresponding Interproscan hits for *A. welwitschiae* proteins are shown below the alignment.

detected in the gene of strain CCMB 663 (ON695834).

In OG4361 (Fig. 2B), three amino acid exchanges were detected; sites 97 and 1292 in strain CBS 139.54 (RDH27708.1), and site 1331 in strain CCMB 674 (ON695839). No mutations were detected in the gene of strain CCMB 663 (ON695835). The mutation in site 97 (non-cytoplasmatic domain) is unique to CBS 139.54, where the typical proline is exchanged for a leucine. The mutation in site 1331 is unique to CCMB 674, in which the typical glycine is exchanged for serine. The mutation in site 1292 is also seen in two strains of *A. niger*, where the glycine is exchanged for an arginine. Both affect the proton exchanger domain, in a non-cytoplasmatic and transmembrane domain, respectively.

2.3. Putative effectors in a. welwitschiae and their adaptive role

The content of effector proteins in *Aspergillus welwitschiae* was described in a workflow of five software aiming to remove non-effector proteins; DeepSig [58], SignalP v4.1 [59], TargetP v1.1 [60], TMHMM v2.0 [61], and EffectorP v3.0 [62]. In total, we identified 660 candidate effector proteins; 231 in *A. welwitschiae* strain CCMB 674, 188 in strain CCMB 663, and 241 candidates in strain CBS 139.54. They were divided into 263 orthogroups, of which 9 were exclusive of CCMB 663 and CBS 139.54 and 1 exclusive of CCMB 663 and CCMB 674 (families containing only genes of these strains). The orthogroups containing candidate effectors were aligned and tested for positive selection evidences, using HyPhy with BUSTED and MEME models as previously mentioned. Our positive selection analysis obtained 12 gene families positively selected, one of which is positively selected in *A. welwitschiae* strains CCMB 663 and CCMB 674, with no orthologs in strain CBS 139.54.

Orthogroup OG1694 was identified as a putative effector protein with positive selection on four amino acid sites (Fig. 3). It contains two CCMB 674 genes, one CCMB 663 gene, and two CBS 139.54 genes. Interproscan identified a signal peptide and a PEBP domain in this family, although neither could be detected on the strain CCMB 674 gene ON695837. Three of these genes were clustered in a clade (ON695838 from CCMB 674, ON695836 from CCMB 663, and RDH30697.1 from CBS 139.54) and the only mutation between them is a change of leucine to phenylalanine in the signal peptide of CBS 149 (gene RDH30697.1). As for the other two genes clustered separately in another clade, ON695837 from CCMB 674 has a gap in the signal peptide, while the pattern in RDH34005.1 is seen elsewhere.

3. Discussion

In this study, we aimed to understand the phylogenetic relationship of *Aspergillus welwitschiae* in comparison with other fungal species, and prospect genes likely involved in the infection, disease progression, and other pathogenicity traits in this species that might help in the epidemiological management of the bole rot of sisal. Our results show that

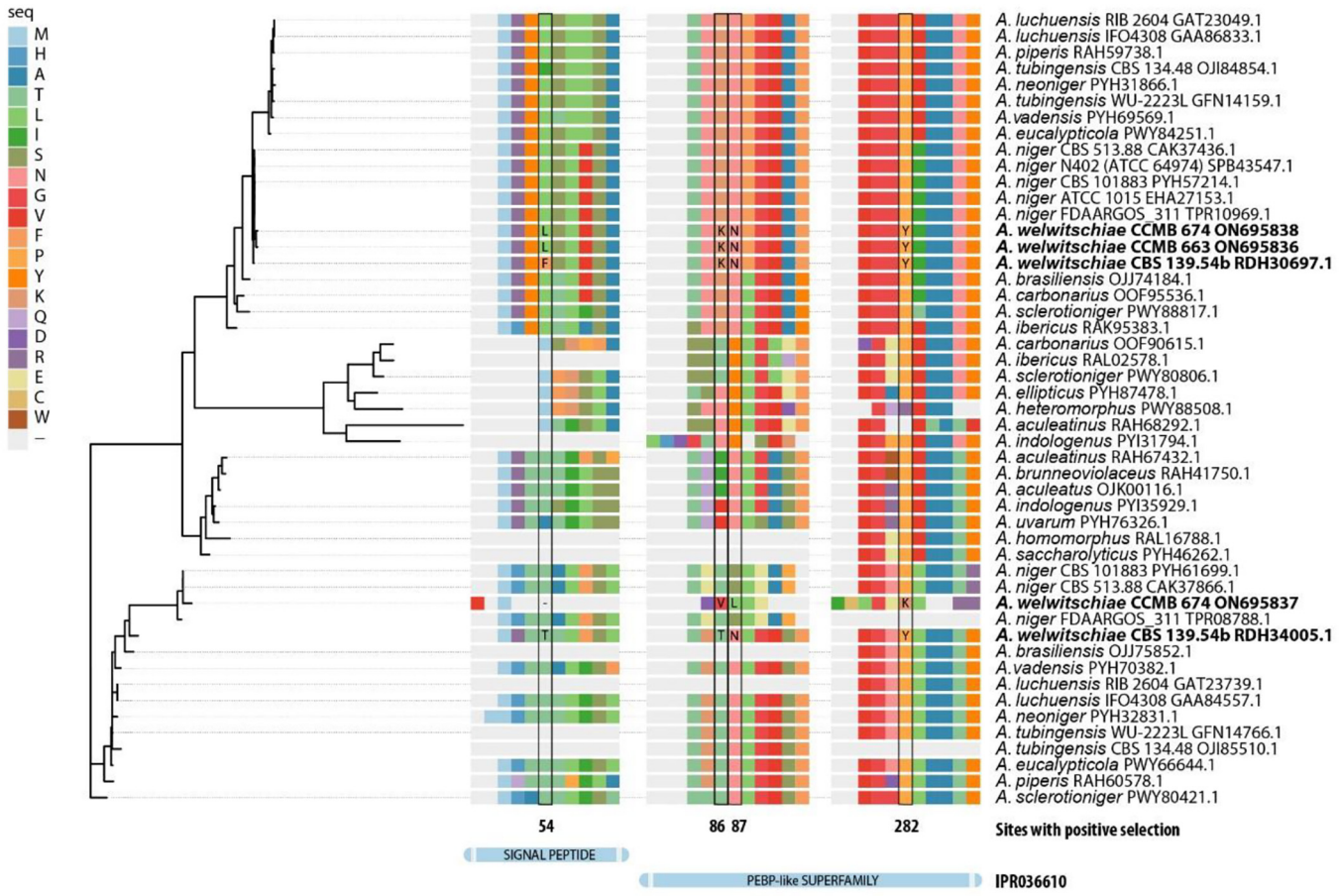

Fig. 3. Sites with positive selection for OG1694, coding a putative effector protein. The figure shows a 10-amino acid region around three alignment sites with positive selection evidence ($p < 0.05$). The corresponding Interproscan hit for *A. welwitschiae* proteins is shown below the alignment.

phylogenomic relationships differ from our early thoughts, indicating *A. welwitschiae* is still not well-defined taxonomically. Investigating the evolution of gene families, we were able to reveal positive selection evidence at the sequence level that might play a role in *A. welwitschiae* pathogenicity. With the appropriate methods, we show that 28 *A. welwitschiae* orthologs differ from all other analyzed species with a *p*-value <0.0001, including those orthologs of *A. niger*. 15 of those orthologs have previously-described roles in pathogenicity. The detection of diversifying selection in these orthologs suggests that such genes are relevant to the metabolism of *A. welwitschiae* in a different form than other species, and when related to pathogenicity, that suggests that these genes have a more crucial role in *A. welwitschiae* than it does in the other species.

Our data shows that the clade including the three strains of *Aspergillus welwitschiae* does not possess a single common ancestor, but it rather looks tangled with its closest, morphological cryptic species, *Aspergillus niger* (Fig. 1). *A. welwitschiae* CCMB 674 escapes the clade containing the other two strains of *A. welwitschiae*, sharing its closest ancestor with the *A. niger* clade instead. Nonetheless, it diverged early from the MRCA relative to the monophyletic *A. niger* clade. It might indicate an ongoing process of speciation, meaning that strain CCMB 674 is a different phylogenetic lineage both from the *A. niger* and *A. welwitschiae*. While this indicates a conflicting identity for *A. welwitschiae* CCMB 674, deeper analysis is required to state whether it is a separate species or not. Giraud et al. (2010) [63] describe that substrate and host affinity might be a condition for this sympatric speciation, as well as mating inside the host plant. Mating; however, remains elusive in *A. welwitschiae, A. niger,* and many other species in this genus [64] since they appear to be asexual/clonal species. Furthermore, both CCMB 674 and CCMB 663 affect the same host (*Agave sisalana*), and *Aspergillus* species, in general have a broad range of hosts and niches, with niche/host switching as a common trait [65–67]. Since *Aspergillus* spores are ubiquitous in the air [64], different species are commonly found in close areas [68,69]. Kollár et al. (2022) [70] reinforce the idea of species as a spatiotemporal entity, rather than a terminal state. According to the authors, the present time state (T_0) is preceded by two separately evolving stories (T_{-1}) which is then preceded by a period of restrictions in gene flow, gradually leading to their divergence (T_{-2}). We hypothesize that *A. welwitschiae* CCMB 674 is between T_{-2} and T_{-1} in comparison to the other strains of *A. niger*. Hence, the driving forces behind the differentiation among *A. welwitschiae* and *A. niger* could be restricted to the genetic drive and the lack of gene flow [64].

Looking at specific gene functions and sequence similarities (Figs. 2–4) gave us a more detailed understanding of how the *A. welwitschiae* strains differ. Our diversifying selection analysis detected different mutations that might confer advantages to various sites in different domains, especially for *A. welwitschiae* CCMB 674, which reinforces the differentiation of this strain, but also for CCMB 663 and CBS 139.54 in some cases. Mutations with the strongest impact on the protein function are those in which the amino acid polarity, charge, or size, is altered. A Glycine is exchanged for a Serine in two sites; in the siderophore domain of OG3308 (Table 1, Fig. 2A) in *A. welwitschiae* CCMB 674 and CCMB 663, and in the Na^+/Ca^{2+} exchanger region of OG4361 (Table 1, Fig. 2B), only in *A. welwitschiae* CCMB 674. Glycine is a nonpolar amino acid while Serine is polar neutral. In the Na^+/Ca^{2+} exchanger region of strain CBS 139.54, a glycine, which is a nonpolar amino acid, is replaced with an Arginine, which is a polar, positively

charged amino acid.

The genes in OG3308 (Fig. 2A) code for proteins containing MFS domains, more specifically for the transport of siderophores (PTHR23501:SF87). Siderophores are iron-chelating compounds, usually small secreted proteins. Saha et al. (2013) [71] pointed out that only the export of siderophores can be achieved via MFS, while the import of Fe ions or the Fe-siderophore complex is usually performed by ABC transporters or permeases. As for pathogenicity, the presence of a siderophore-based iron acquisition system is typical of pathogenic microorganisms, and a requirement for successful pathogenicity [72]. The positive selection/selective sweep of fungal MFS transporters is described in a few articles, such as in *Rhynchosporium commune* [73], *Sclerotinia sclerotiorum* [74], and *Zymoseptoria tritici* [75]. Nonetheless, none of these works go into detail about the modifications of these transporters.

Genes in OG4361 (Fig. 2B) code for a calcium efflux pump; the Na^{+}/Ca^{2+} exchanger protein. In *Cryptococcus neoformans*, a mutation in a similar protein disrupts the Ca^{2+}-calcineurin signaling pathway, decreasing pathogenicity (measured by survival rate in contact with mice macrophages). This mutation also increased sensitivity to Ca^{2+} ions under $CaCl_2$ stress [44]. Nonetheless, in plants, $CaCl_2$ is related to drought resistance [76,77] but it might also cause osmotic stress [78]. For this reason, although Kmetzsch et al. (2010) [44] describe an ortholog of their exchanger protein in *A. fumigatus*, the role of such protein in plant pathogens such as *A. welwitschiae* is still unclear.

A set of mutations are also present in effector genes (OG1694, Fig. 3), especially in *A. welwitschiae* CCMB 674 (Fig. 3). Except for a mutation in the signal peptide, in which leucine is exchanged for phenylalanine in *A. welwitschiae* CBS 139.54, all other sites refer to the gene ON695837 of strain CCMB 674. In this gene, no signal peptide was detected, nor was the PEBP domain found in other genes, which might indicate that this effector gene is prone to duplications and mutation, as so happens in other effector genes [79–81]. This gene was likely included in this gene family because it shares similarities with the other genes later in the sequence, in an area not shown in the positively selected sites. Therefore, we hypothesize that this specific gene could be originated from an event of duplication, and following the theory of birth-and-death of gene evolution [79], it might have a different function (since the PEBP domain is undetectable) or be affected by consecutive mutations to the point it becomes completely uncharacterized. In fungi, PEBP has been described as a protease inhibitor [82,83]. In plants, however, it is well-studied as a florigen [84–86]. As it is a secreted protein, we believe that fungi-synthesized PEPB could mimic plant PEBP and induce the transition from vegetative to reproductive states. In *Agave,* flowering mobilizes fructan polymers, degrading complex structures to oligofructans [87]. Hence, we hypothesize that fungal PEBP could trigger fructan degradation typical of flowering stages in *Agave* to then feed off simpler sugar molecules that would be addressed to the flowering structures.

Among other gene families previously described as participating in pathogenicity, we found some with positive selection in *A. welwitschiae* (Table 1). Family OG3525 includes proteins in the APSES-type DNA-binding domain superfamily and KilA-N and Ankyrin repeats. APSES proteins are transcription factors in the topology HTH (winged helix-turn-helix family of proteins). They commonly contain KilA-N domain, described in the literature as laterally transferred from DNA viruses [88] and Ankyrin repeats. Despite their broad spectrum of functions, they have been described as regulators of the production of secondary metabolites in some fungal species. In *Ustilago maydis*, an APSES-type protein was shown to be increasingly expressed 14 days after infection in corn, repressing the formation of teliospores (spores that give rise to basidia) during the biotrophic stage [31]. APSES-mutants in *Fusarium verticilioides* were shown to decrease the production of fumonisin and fusarin C, and decrease the efficiency in the infection of maize seedlings [33]. In the entomopathogenic fungal species *Metarhizium rileyi*, the mutation of APSES-like transcription factors impaired the pathogenicity and increased the sensitivity to thermal stress [34]. Similarly, family OG4087 was annotated as an MBF transcription factor complex, containing the subunit Mbp1/Res1/Res2, which also derives from viral KilA-N repeats [39] and also presents Ankyrin domains. Despite the scarcity of literature exploring MBF in pathogenicity, it has been described that this transcription factor is crucial during the transition from cell division stages G1 to S [40], which indicates that MBF might have similar effects to APSES. Although *A. welwitschiae* does not produce asci (the Ascomycota meiosporangia, homologous to Basidiomycota basidia) or any kind of sexual sporoma (in fact, mating in *A. welwitschiae* remains undescribed), fumonisins or fusarin C, as seen in our 2019 study [19], suggests that these protein domains and families are involved in lifestyle switching and the production of secondary metabolites.

Various gene families detected to be positively selected in our *A. welwitschiae* strains might operate on pathogenicity via the metabolism of lipids and regulation of the fungal cell wall (Table 1). The component PIL1/LSP1 is included in OG3961, and the protein coded by these genes is part of the eisosome, a domain of the plasmatic membrane with diverse functions, including regulation of sphingolipids and lipid homeostasis as well as cell wall morphogenesis [89–91]. PIL1/LSP1 forms filaments that bend the membrane into the typical furrow shape of the eisosome [89]. In the genus *Aspergillus*, there are a few studies on the role of the eisosome on conidiation in *Aspergillus nidulans* [36,92,93] and *Aspergillus fumigatus* [35]. Nonetheless, some research in fungal species phylogenetically distant from *A. welwitschiae* describe the role of eisosomes in *Alternaria brassicicola* [38] and *Beauveria bassiana* [37]. In *Alternaria brassicicola*, Colou et al. [38] have described that eisosome components are overexpressed when the spores were exposed to common plant defense mechanisms (phytoalexins, osmotic stress); however, mutations in Pil1A and Pil1B did not increase susceptibility to those conditions. The authors [38] then hypothesize that eisosomes are involved in appressoria formation, a structure used to invade the plant host cell, usurp nutrients and deliver toxic compounds. Appressoria were never described in *A. welwitschiae*, and a study including *A. fumigatus* [94] could not find this structure in this species either, although that does not harm their ability to invade the host cells. In *B. bassiana*, an entomopathogen, mutations in Pil1A and Pil1B have disputed the expression of autophagy-related genes, the formation of autophagosomes, and decreased pathogenicity by making the fungus unable to deliver proteases into the host, which could be viewed as an equivalent to the eisosome role in toxin delivery via appressoria [37].

Another family with positive selection and related to lipid metabolism was annotated as AGC Kinase. The role of AGC Kinases in the metabolism of sphingolipids and pathogenicity have been described in the literature. While in *A. fumigatus* the silencing of ypkA, an AGC Kinase, causes a series of growth defects [55], its mutation also causes decreased pathogenicity in plant and insect fungal pathogens [53,54]. The SAC1 protein (OG4394) and the Ras-like guanine nucleotide exchange factor (OG4981) might also play a role in pathogenicity via the maintenance of cell wall integrity, biofilm formation, and chitin deposition [49,56,57]. Still from the perspective of lipid metabolism and appressoria formation, the lipases (OG4836) act as important pathogenicity factors in fungi, both for human pathogens [50,51] and for plant pathogens [95]. In the latter, lipases were shown to be highly expressed in early infections, while their repression significantly decreases disease progression.

4. Conclusions

In this paper, we performed a comparative genomics study of 40 *Aspergillus* species and 2 outgroups, including three strains of *A. welwitschiae*, a saprotroph/opportunistic pathogen. Among these, two strains were isolated from *Agave sisalana* affected with the bole rot disease (CCMB 663 and CCMB 674). Previous research of our research group [13] indicated that these two strains belong to two different haplotypes, but our current study shows that CCMB 674 shared its last common ancestor with *A. niger*, while its last common ancestor with the

clade including CCMB 663 dates back to 833 thousand years ago. We found point mutations in the genomes of *A. welwitschiae* that could be related to pathogenicity. These mutations affect both primary metabolism genes, such as membrane transporters and efflux pumps, and specific traits, such as effector proteins that might disrupt sugar metabolism in the host plant. With these results, we hope to expand the knowledge on the evolution of the *Aspergillus* genus and improve disease management through the dispersion of healthy individuals. *Aspergillus welwitschiae* is a saprotroph/opportunistic pathogen.

5. Materials and methods

5.1. Genomic data

In addition to various species in the sections Nigri and Flavi of the genus *Aspergillus* and other fungal species, which are listed in detail in Table S1, this study comprises a new strain of *Aspergillus welwitschiae*: CCMB 663, which was directly isolated from the stem of a field-collected *Agave sisalana* plant in Conceição do Coité, State of Bahia, Brazil, and belongs to the same haplotype as *A. welwitschiae* strain CBS 139.54, as determined in Duarte et al., (2018) [13]. Briefly, the stem sample from which CCMB 663 was obtained was surface-sterilized and then plated on PDA with chloramphenicol. *Aspergillus* CFUs were then cultivated onto a new PDA plate. The identity of this strain was confirmed through Sanger sequencing, and haplotypes were determined with adequate statistical tests using all available *A. welwitschiae* barcoding sequences (calmodulin gene) in public databases. For the WGS, the mycelia of *A. welwitschiae* strain CCMB 663 was grown on potato dextrose agar medium (PDA – Sigma-Aldrich, Missouri, USA) and incubated at 25 °C for 5 days, or until covering the surface of a 9-cm Petri dish. Subsequently, the mycelium was scrapped for genomic DNA extraction, which was performed with FastDNA™ for Soil kit (MP Biomedicals – California, USA). Genomic DNA quality and quantity were assessed by agarose gel electrophoresis and fluorometric analysis, respectively. A 450-bp library was prepared with NEBNext Fast DNA Fragmentation and Library Preparation Kit (New England Biolabs, Nebraska, USA) following the manufacturer's instructions. Library quality was evaluated with a 2100 Bioanalyzer (Agilent – California, USA), and whole-genome sequencing was performed on a HiSeq 2500 platform with the paired-end strategy and estimated fragment size of 450 bp (Illumina – California, USA) in the Georg August University Göttingen (Göttingen, Germany).

5.2. Genome assembly, annotation, and quality control of Aspergillus welwitschiae CCMB 663

Genome assembly for *Aspergillus welwitschiae* strain CCMB 663 was performed as in Quintanilha-Peixoto et al., (2019) [19]. Briefly, raw reads were trimmed (Phred score > 20) with BBDuk and normalized with BBNorm, both being components of the BBTools v.36.86 software package [96]. Normalized reads were assembled with SPAdes v. 3.11.1, and assembly metrics were assessed using the Perl script scaffold_stats.pl [97] and BUSCO v. 3.1 [98]. Short contigs (< 800 nt) were removed without any impact on ORF (open reading frame, coding) content (according to BUSCO results). Non-annotated scaffolds of *A. welwitschiae* CBS 139.54 were obtained from the Joint Genome Institute portal (JGI; https://jgi.doe.gov/). Assembly QC was analyzed with Perl script scaffold_stats.pl and BUSCO v.3, using the Ascomycota lineage dataset. The resulting assemblies were annotated with MAKER2 v2.31.9 [99], with support from ab initio predictors SNAP [100], GeneMark [101], and Augustus [102], as seen in Masonbrink et al. (2019) [103]. To improve gene prediction, a protein set containing sequences from *Aspergillus welwitschiae* and *Aspergillus niger*, the phylogenetically closest species to *Aspergillus welwitschiae* [16], was also used.

5.3. Effector protein detection

Our approach to identifying secreted proteins was modified from Cuesta-Astroz et al. [104]. All annotated proteins in *Aspergillus welwitschiae* strains CBS 139.54, CCMB 663, and CCMB 674 were submitted to DeepSig [58] and SignalP v4.1 [59], both of which identify classical secretory proteins. Following that, possible mitochondrial proteins were removed by using TargetP v1.1 [60], and sequences containing transmembrane domains were removed using TMHMM v2.0 [61], using default parameters for the three applications. For our effector proteins analysis, it is worth noting that we considered classical effectors, that is, proteins acting directly on the host, addressed outside of the fungal cell via a signal peptide. The three predicted secretomes were submitted to EffectorP v3.0 [62]. Putative effectors were also annotated with Localizer (for the prediction of subcellular localization in the host plant). Finally, candidate effectors were annotated with InterPro and Pfam using GO Feat [24].

5.4. Homology assignment and phylogenetic inferences

Homology assignment of gene families was performed using OrthoFinder v2.5.2 [105]. The species used in this analysis comprise the three aforementioned strains of *A. welwitschiae*, 37 other *Aspergillus* species from sections Nigri, Flavi, and other sections, as well as *Neurospora crassa* and *Penicillium rubens* as outgroups, all with public genomes (Table S1). The species phylogenomics was inferred using 1842 single-copy orthologous gene families, whose proteins were aligned using the built-in MAFFT module in OrthoFinder with default parameters and then concatenated in a supermatrix. We used IQ-TREE v. 2.1.2 [106] for phylogenetic inference with the parameter "-m TEST" to calculate the best fitting model using BIC criteria, which was JTT + F + I + G4, with 1000 ultra-fast bootstrap replicates for branch support. IQ-TREE was also used for the time calibration of the tree with the least-squares analysis and two calibration points obtained from the TimeTree database [107]: 64 Mya for the *Aspergillus* genera MRCA, and 3.8 Mya for *A. oryzae* and *A. flavus* MRCA. For the phylogenetic inference of individual gene families including paralogs, we used PRANK v.150803 [108] for the alignment using the parameter "-codon", and, to obtain the alignments in protein sequences, we used the parameters "-translate" and "-convert". IQTREE was used with the same settings as aforementioned.

5.5. Detection of positive selection and expression in the predicted coding sequences

For all single-copy gene families, we searched for evidence of episodic positive (diversifying) selection using the BUSTED model [22] implemented in HyPhy v.2.5.32 [21]. Families where a positive selection was detected with p-values <0.001, were also tested by the branch-test of the MEME model [22] implemented in HyPhy, using genes from the three *A. welwitschiae* strains marked as the foreground to be contrasted with the rest of the tree.

Availability of data and materials

The datasets generated and analyzed during the current study are available in the GenBank repository, under accession codes ON695834-ON695840.

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CRediT authorship contribution statement

Gabriel Quintanilha-Peixoto: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Marina Püpke Marone:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Fábio Trigo Raya:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Juliana José:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Adriele Oliveira:** Methodology, Validation, Formal analysis. **Paula Luize Camargos Fonseca:** Methodology, Investigation. **Luiz Marcelo Ribeiro Tomé:** Methodology, Investigation. **Dener Eduardo Bortolini:** Software. **Rodrigo Bentes Kato:** Methodology. **Daniel S. Araújo:** Formal analysis. **Ruth B. De-Paula:** Formal analysis. **Yesid Cuesta-Astroz:** Software. **Elizabeth A.A. Duarte:** Formal analysis. **Fernanda Badotti:** Conceptualization. **Vasco Ariston de Carvalho Azevedo:** Resources, Funding acquisition. **Bertram Brenig:** Resources, Funding acquisition. **Ana Cristina Fermino Soares:** Resources, Project administration, Funding acquisition. **Marcelo Falsarella Carazzolle:** Resources. **Gonçalo Amarante Guimarães Pereira:** Resources, Project administration, Funding acquisition. **Eric Roberto Guimarães Rocha Aguiar:** Conceptualization, Supervision. **Aristóteles Góes-Neto:** Conceptualization, Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

None of the authors have any competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2022.110517.

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