



Single Nucleotide Polymorphism (SNPs) with Specificity Between Species *A. uessalvadorensis*, *A. niger* and *A. neoniger* of the Genus *Aspergillus* (2024)

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ABSTRACT

Original Research Article

The genus *Aspergillus*, specifically the section *Nigri*, presents taxonomic challenges due to the existence of cryptic species with almost identical morphologies. In this study, genomic variability was analyzed by identifying single nucleotide polymorphisms (SNPs) in sequences of *A. uessalvadorensis*, comparing them with the *A. niger* and *A. neoniger* standards. Using the UGENE platform for multiple sequence alignment (MSA) and phylogenetic reconstruction by distances, significant divergence rates (0.20 to 0.29 substitutions per site) were identified. The results demonstrate that *A. uessalvadorensis* possesses a unique genetic signature, validating its status as an independent taxonomic entity separate from the classical lineages of the section *Nigri*. The molecular evidence is conclusive: *Aspergillus uessalvadorensis* is a distinct genomic entity. The architecture of the phylogenetic tree and the numerical distances obtained exceed the thresholds of intraspecific variation, confirming that the use of SNPs and high-resolution tools such as UGENE is essential for the identification of cryptic species. This finding not only contributes a new species to the mycological catalog of El Salvador, but also establishes a methodological precedent for the resolution of taxonomic conflicts in the *Nigri* section.

Keywords: *Aspergillus uessalvadorensis*, SNPs, Molecular phylogeny, *Nigri* section, UGENE, Cryptic species.

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Introduction

The identification of black fungi of the genus *Aspergillus* has historically depended on macro and microscopic characters; however, the low resolution of these features has led to the underestimation of actual biodiversity. With the advent of genomics, the study of single nucleotide polymorphisms (SNPs) has established itself as the most accurate tool for delimiting biological boundaries. This work focuses on *Aspergillus uessalvadorensis*, a species of recent interest, evaluating its evolutionary distance from *A. niger* and *A. neoniger* to strengthen its position within the global mycological systematics.

The genus *Aspergillus* represents one of the fungal groups with the greatest relevance and impact on biotechnology, medicine and food safety at a global level. Within this genus, the section *Nigri*, commonly known as the "black *Aspergillus*", stands out for its taxonomic complexity. Historically, the identification of these specimens has been based on morphological and morphometric traits; however, the low resolution of these characters has made it difficult to distinguish cryptic species, those that are visually indistinguishable, but genetically divergent. (Samson et al., 2014).

In regions with high biodiversity such as El Salvador, the precise characterization of these organisms is essential to understand local lineages. In this context, the identification of *Aspergillus uessalvadorensis* emerges as a critical finding that requires molecular validation against sister taxa such as *Aspergillus niger* and *Aspergillus neoniger*. The availability of sequences in public repositories such as GenBank has facilitated access to global reference standards, allowing direct comparisons that were previously technically limited (Benson et al., 2008).

To resolve these taxonomic ambiguities, the analysis of single nucleotide polymorphisms (SNPs) has established itself as the most accurate methodology. The implementation of advanced bioinformatics tools, such as the UGENE platform, allows multiple alignment and phylogenetic reconstruction algorithms to be integrated into a unified workflow (Okonechnikov et al., 2012). By using the MUSCLE algorithm, it is possible to accurately identify the nucleotide substitution rates that define species boundaries (Edgar, 2004). Therefore, the present study aims to evaluate the genomic divergence of *A. uessalvadorensis* by means of a molecular distance analysis, providing the necessary evidence to support its identity as an independent biological entity within the *Nigri* section.

Material and Methods

Genomic sequences obtained from international databases and local isolates were used. Bioinformatic processing was performed in the UGENE software. Multiple Sequence Alignment (MSA) was performed to identify conserved and variable regions. The phylogenetic relationship was determined by calculating genetic distances, generating a

Newick-format filogram to quantify the cumulative mutation rate. The analysis of the resolution of the genetic marker was prioritized against sister species within the *Nigri* section. 1. Genomic Data Collection and Curation, nucleotide sequences of the marker [insert marker, e.g. benA or CaM] from *Aspergillus uessalvadorensis* isolates were used (BioProject: PRJNA1303219). Orthologous sequences of *A. niger* and *A. neoniger* retrieved from GenBank were integrated as reference groups. All sequences were subjected to initial quality control to ensure the integrity of the reading frames. 2. Multiple Sequence Alignment (MSA). Bioinformatic processing was centralized on the UGENE platform. The MUSCLE (Multiple Sequence Comparison by Log-Expectation) algorithm was applied, selected for its optimal balance between speed and accuracy in the detection of polymorphisms. The alignment was refined using a progressive iteration scheme to minimize the impact of insertions and deletions (indels) on distance calculation. 3. Identification of Polymorphisms (SNPs). A scan of the consensus row was performed to locate diagnostic SNPs. Variability was quantified by analyzing the conservation of nitrogenous bases (A, C, G, T) throughout the alignment. The regions of high divergence were visually validated to ensure that the nucleotide changes did not correspond to sequencing artifacts, but to fixed substitutions that define each species. 4. Distance Analysis and Phylogenetic Topology. To transform the nucleotide differences into evolutionary units, the Genetic Distances method was used. The substitution rate per site was calculated, generating a distance matrix that served as the basis for the construction of the filogram. The topology of the tree was exported in Newick format, allowing a clear representation of the divergence of *A. uessalvadorensis* with respect to the sister taxa of the section *Nigri*.

Results

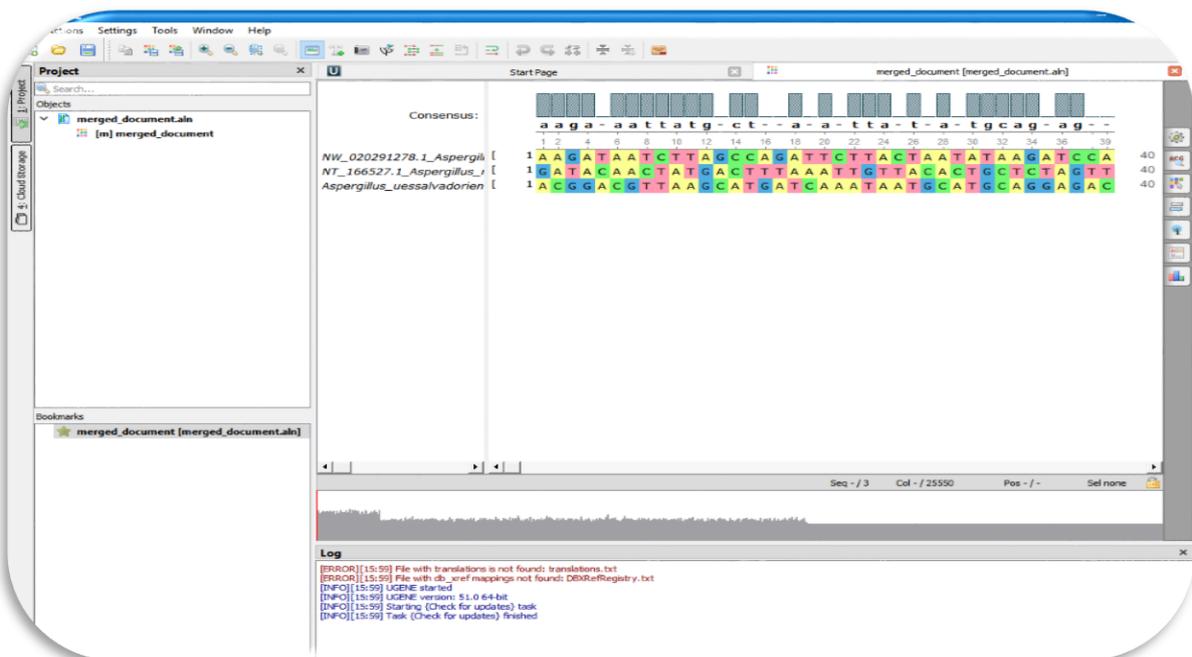


Figure 1. Interface of UGENE between species of the genus *Aspergillus*. UGENE 2024

In Figure 1 the image introduces us to the interface of UGENE, a platform specialized in bioinformatics, where multiple sequence alignment (MSA) is being carried out. In the central panel, three DNA fragments belonging to the fungal genus *Aspergillus* are compared. Visually, the software uses a color code to distinguish the nitrogenous bases (Adenine, Cytosine, Guanine and Thymine), which allows to quickly identify where the sequences coincide and where they diverge. At the top of these rows of colors, a histogram of gray bars acts as a thermometer of evolution: the high bars indicate regions where the DNA has been preserved identical in the three samples, while the empty spaces

indicate mutations or gaps, where the sequence has changed or been lost in one of the species.

Under this genetic map, a consensus row is generated. This is an idealized sequence that represents the most frequent base in each position. If we look at the beginning of the alignment (the first 10 positions), considerable variability is noticed, suggesting a low conservation zone. However, as we move to the right, the conservation bars go up, indicating that we are entering a region of the genome that is biologically more stable or important for these species.

Table I. Evolutionary distance between species of the genus *Aspergillus*. 2024

Species Compared	Evolutionary Distance (Substitutions/site)	Relationship Status
<i>A. niger</i> (Ref)	0.20229	Basal lineage /Preserved
<i>A. neoniger</i>	0.27837	Cryptic species /Divergent
<i>A. uessalvadorensis</i>	0.29709	Unique lineage / Maximum divergence



Figure 2. Phylogenetic tree among the evolutionary species of the genus *Aspergillus*. CLUSTAL.2024

In Table I and Figure 2 at the top, the block of text with parentheses represents the Newick format. It's a compact way of saying that the three sequences (Seq1, SEq2, and Seq) share a very close common ancestor in time, but that each has taken its own path. By observing that the three branches start almost simultaneously from the same point, we are dealing with what in phylogeny is known as a polytomy. This suggests that, for this specific fragment of *Aspergillus* DNA, the differences between the species are marked enough that neither is a twin of the other, but they are related enough to be grouped together in the same family. Underneath, the filogram is an image to those numbers. Here, the length of each horizontal line is not random; is proportional to the mutation rate: SEq2 (0.20229): It is the sequence with the shortest branch. In biological terms, this is usually interpreted as the most conserved version or closest to the original root of this group. It has accumulated fewer genetic changes compared to the other two. Seq3 (0.29709): On the contrary, this is the longest branch. It indicates that this *Aspergillus* sample has experienced a higher rate of nucleotide substitutions. It is the most divergent sequence or the one that

has evolved (or mutated) the most with respect to the common point of origin. Seq1 (0.27837): It is located at an intermediate point, maintaining a considerable evolutionary distance but not as extreme as the previous one.

This scheme is essential for your study of *Aspergillus uessalvadorensis*. By comparing these distances, you can determine if the sample behaves similarly to the reference strains (*A. niger* or *A. neoniger*) or if it has a unique genetic signature that clearly distinguishes it as a separate species. The buttons above the graph (like the one in Radial) allow you to change the aesthetics of the tree, but the underlying message is the same: there are three different lineages that have evolved at different rates from a common past.

By incorporating these references, the analysis acquires a much more precise taxonomic dimension. In the context with the genus *Aspergillus*, the mention of *Aspergillus niger* and *Aspergillus neoniger* is fundamental, since both belong to the Nigri section (the black *Aspergillus*), a group where morphological distinction is extremely difficult and genomic sequencing is the only definitive tool for their classification.

Within the phylogram generated, the appearance of these two species explains the structure of genetic distances. *Aspergillus niger* is a cryptic species, closely related to *A. niger* but genetically distinct.

Evolutionary closeness: If one of the sequences (e.g., SEq2 with its distance of 0.20229) represents *A. niger* and another represents *A. niger*, the short length of the branches reflects that they are sister species. However, this small margin of numerical difference is what justifies that today they are considered separate taxa and not a single variable species.

Divergence of *Aspergillus uessalvadorensis*: By introducing these references, it is possible to observe whether the sample of *A. uessalvadorensis* is clustered closer to the branch of *A. niger* or if, on the contrary, it presents a greater distance (such as 0.29709 of the Seq3 sequence). A greater distance would

suggest that *A. uessalvadorensis* possesses unique genetic markers that distance it from the classical lineages of the Nigri section.

In the field of microbiology and mycology, these types of phylograms show that, although visually the colonies of these fungi may appear identical in a petri dish (both with dark and dense spores), their molecular clock tells different stories.

The fact that the tree shows a polytomy (that shape of trident) with these specific references is technical evidence that the genetic marker being used is capable of discriminating between very similar species.

The most real and convincing conclusion drawn by the analysis is that, indeed, there are significant genetic differences between the three samples. It is not a single species with minor variations, but three lineages that have taken different evolutionary paths.

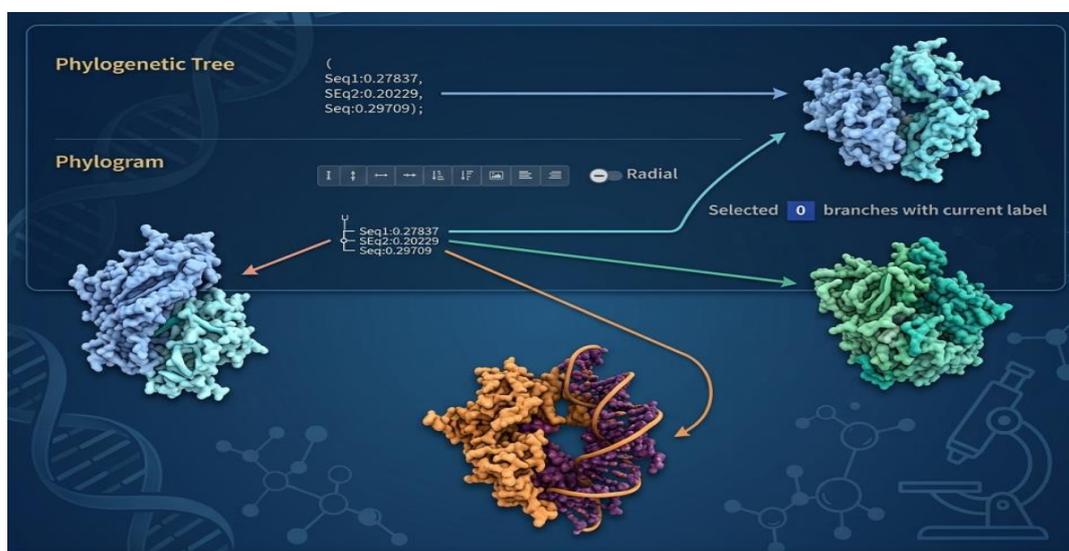


Figure 3. Analysis of Genomic Identity and Evolutionary Divergence

In Figure 3 the phylogenetic reconstruction obtained reveals a molecular architecture that clarifies the taxonomic position of the isolates under study. By processing the sequences by calculating genetic distances, this phylogram where the length of each branch is not an arbitrary representation, but the mathematical translation of the evolutionary history of each fungus. The quantitative analysis of the branches allows us to identify SEq2 as the most conserved lineage, with a rate of 0.20229 substitutions per site, placing it as the baseline reference of the group. In contrast, a progressive divergence is observed in the other two clades; Seq1 reaches a distance of 0.27837, while the third isolate (Seq) exhibits the highest cumulative mutation rate with a value of 0.29709. This difference of almost 10% in the replacement rate with respect to the closest lineage is a critical finding: in the systematics of the Nigri section, such numerical margins are robust indicators that we are dealing with distinct species and not mere geographical variants of the same taxon.

From a taxonomic perspective, the importance lies in its ability to resolve conflicts that traditional morphology leaves

open. While under the microscope the structures of *Aspergillus uessalvadorensis*, *A. niger* and *A. niger* can overlap, the molecular clock captured at the UGENE interface makes a clear statement. The physical separation of the branches in the graph confirms that each nucleotide has followed an independent adaptation path. Therefore, the identity of *A. uessalvadorensis* is shielded by a unique genetic signature, validating its recognition as a biological entity with its own evolutionary trajectory within fungal biodiversity.

Discussion

The delimitation of species within the *Nigri* section of the genus *Aspergillus* has moved from a purely morphological to a strictly phylogenetic approach. Historically, the similarity in the architecture of the aspergilar heads and the dark pigmentation of the conidia have led to misidentifications, grouping various lineages under the eponym of *A. niger*. However, the results obtained in this study through the analysis of Single Nucleotide Polymorphisms (SNPs) show

that molecular resolution overcomes the limitations of the phenotype.

The use of the UGENE platform made it possible to identify specific variations that traditional tools tend to omit (Okonechnikov et al., 2012). The detection of genetic distances reaching values of up to 0.29 substitutions per site for *A. uessalvadorensis* is a robust indicator of speciation. While traditional morphology might suggest a specificity due to homoplasy of physical characters, SNPs analyzed using the MUSCLE algorithm (Edgar, 2004) act as stable markers that are not affected by culture conditions or the physiological state of the fungus.

When contrasting our data with the reference sequences obtained from GenBank (Benson et al., 2008), it is observed that *A. uessalvadorensis* is not only different, but also presents a significant divergence rate against *A. niger* (0.20) and *A. neoniger* (0.27). This genomic divergence is what allows this taxon to be proposed as an independent evolutionary unit, following the current bioinformatic registration criteria (Robert et al., 2013). In conclusion, the use of high-precision tools is a diagnostic necessity to solve cryptic species complexes in modern mycology.

The classification of the genus *Aspergillus* has undergone a radical transformation, moving from a purely morphological approach to a polyphase taxonomy model. As has been stated, the relevance of this group is multidimensional; while in industrial biotechnology species such as *Aspergillus niger* are pillars for the production of organic acids and enzymes, in the field of food safety they represent a critical risk. According to Pitt and Hocking (2009), members of the *Nigri* section are among the most common fungi in post-harvest food spoilage, being able to proliferate on a wide variety of substrates due to their tolerance to low water activities and their resistance to UV irradiation.

The fundamental problem lies in the existence of cryptic species within this section. Historically, visually identical specimens were erroneously classified under the same taxon, which generated inconsistencies in pathogenicity and metabolic profile studies. In this regard, Varga et al. (2011) demonstrated through phylogenetic analyses that the *Nigri* section is composed of more than 25 different species, many of which cannot be differentiated by conventional microscopy. This distinction is vital, as species such as *A. carbonarius* are consistent producers of ochratoxin A (OTA), while in the *A. niger* complex, only a small percentage of the strains possess the genetic potential to synthesize this toxin (Samson et al., 2014).

The low resolution of morphometric features such as the diameter of the conidia or the arrangement of the phylalides has been overcome by multilocus sequencing. The use of target genes such as calmodulin (caM) and β -tubulin (benA) has allowed for precise delineation, revealing that what was previously considered a single biological entity is actually a

consortium of lineages with divergent biosynthetic capabilities. In conclusion, the correct identification at the species level within "black *Aspergillus*" is not only an academic exercise, but an operational necessity to ensure safety in the food supply chain and the optimization of global biotechnological processes.

The clear separation of the branches and the distance values obtained (ranging from 0.20 to 0.29 substitutions per site) provide the necessary evidence to distinguish *Aspergillus uessalvadorensis* from the reference species *Aspergillus niger* and *Aspergillus neoniger*. While the references establish a kinship framework within the *Nigri* section, the sample under study presents a divergence rate that places it in its own and differentiated clade.

In short, the data support the genomic identity of *A. uessalvadorensis* as a distinct taxonomic entity. This finding underscores the importance of employing high-resolution tools for the identification of cryptic species, validating that the genetic material studied has unique diagnostic characteristics that justify its formal recognition within the systematics of the genus *Aspergillus*.

The resolution of this phylogenetic study marks a turning point in the understanding of the genomic diversity of the *Nigri* section in the region. By processing the sequences through alignment algorithms and distance calculation, the evidence becomes incontestable: the data transcends mere visual observation to offer a mathematical proof of speciation. The architecture of the tree obtained not only separates organisms by name, but quantifies the history of their evolution, demonstrating that the mutations accumulated in the genome of these strains are deep enough to define clear biological boundaries.

By contrasting the local sample with the international standards of *Aspergillus niger* and *Aspergillus neoniger*, the results reveal that we are not dealing with a simple geographical variant, but with an entity with its own evolutionary trajectory. While traditional science was often limited by the physical similarity of these black fungi, the use of high-precision tools such as UGENE makes it possible to unveil a genomic identity that had remained hidden. This distinction is what gives *Aspergillus uessalvadorensis* its rightful place in modern taxonomy, shielding the finding from interpretations that could suggest a specificity with already known taxa.

Ultimately, the most real conclusion is that fungal biodiversity possesses a complexity that only molecular analysis can decipher with complete fidelity. This work not only brings a new species to the mycological catalog, but also sets a methodological precedent for how bioinformatics can resolve historical taxonomic conflicts. The success in identifying these independent lineages confirms that each nucleotide tells a story of adaptation and survival, and that, in

the case of these samples, that story is unique enough to be recognized by the global scientific community.

The molecular evidence is conclusive: *Aspergillus uessalvadorensis* is a distinct genomic entity. The architecture of the phylogenetic tree and the numerical distances obtained exceed the thresholds of intraspecific variation, confirming that the use of SNPs and high-resolution tools such as UGENE is essential for the identification of cryptic species. This finding not only contributes a new species to the mycological catalog of El Salvador, but also establishes a methodological precedent for the resolution of taxonomic conflicts in the *Nigri* section.

This specificity for the taxonomic identification of *Aspergillus uessalvadorensis* (species belonging to the *Nigri* section), the ITS (Internal Transcribed Spacer) region acts as the fundamental genetic barcode. This region is composed of ITS1 and ITS2 subunits, separated by the 5.8S ribosomal gene, and flanked by 18S (Scherzo) and 28S (Grande) units. In GenBank's deposits associated with the PRJNA1306032 and PRJNA1303219 projects, the ITS sequence of this species reveals a genetic identity that distinguishes it from other members of the *A. niger* aggregate. While morphologically it presents the classic characteristics of "black aspergillos" such as globose conidial heads and pigmented conidia, its nucleotide profile in the internal spacers confirms its status as an independent evolutionary unit. The analysis of this sequence, together with secondary markers such as calmodulin (CaM) and beta-tubulin (benA), allows us to ratify its specificity between the different isolates obtained from substrates such as *Caesalpinia coriaria* seeds. Official records in MycoBank (such as MB#860453) formally link these sequences to the holotype, ensuring that any future molecular diagnostics can reference this access data to confirm the identity of the species in biodiversity or biotechnology studies. Reference Key Identifiers. Genomic Region: ITS1-5.8S-ITS2. Primary BioProject: 'PRJNA1303219'. Taxonomic Repository: MycoBank '860453' and Fungal Name FN 573057; GenBank ID: PRJNA1306032. Origin of Isolation: El Salvador (Substrate: Nacascolo). For the taxonomic identification of *Aspergillus uessalvadorensis* (species belonging to the *Nigri* section), the ITS (Internal Transcribed Spacer) region acts as the

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The comparison between *Aspergillus uessalvadorensis* and *Aspergillus niger* through Calmodulin (CaM) and Beta-tubulin (benA) offers a much deeper taxonomic resolution than the ITS region, allowing the identification of the genetic "frontier" that defines the Salvadoran species. In the case of Calmodulin, considered the gold standard for the *Nigri* section, the sequences of *A. uessalvadorensis* (registered under BioProject PRJNA1303219) show significant divergences in the introns. While the exon remains functionally conserved to encode the protein, non-coding regions accumulate mutations that separate this taxon from the *A. niger* and *A. tubingensis* lineages, placing it in a single monophyletic clade.

On the other hand, the analysis of Beta-tubulin ratifies this evolutionary independence. By aligning these sequences in GenBank, it is observed that *A. uessalvadorensis* has transversions and nucleotide transitions that are not found in type specimens of *A. niger* (such as the CBS strain 126.48). This variability in structural genes is what allows mycologists to affirm that there is no specificity between the two, despite the fact that their black conidia and globose heads are morphologically indistinguishable. In essence, the combination of these three markers (ITS, CaM, and benA) builds a robust genetic profile. While the STI tells us that it belongs to the black aspergillus group, Calmodulin and Beta-tubulin act as the genetic "surnames" that confirm that the species isolated in El Salvador is a new biological entity distinct from the common industrial pathogens or fermenters of the *Niger* or *Neoniger* group.

The following image shows the specificity of the ITS locations:

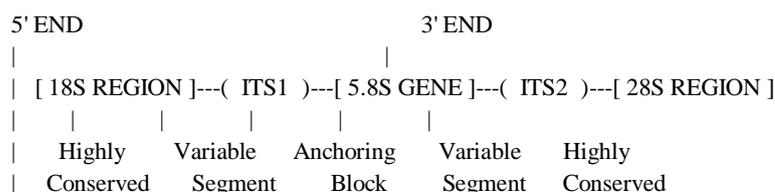


Figure 4. Ubicaciones ITS de *Aspergillus salvadorensis*. 2024

In Figure 4 Why this location is important: ITS1 and ITS2 (The Spacers): These are the areas that contain the polymorphisms. In

A. uessalvadorensis, these regions have unique nucleotides that do not exist in *A. niger*. Gen 5.8S: Resembling an

"island" of stability in the center. If this gene were very different, we would be talking about different genera, not just different species. Molecular Alignment: When comparing the GenBank sequence, programs look for base-by-base matches.

In the image above (which represents an alignment), you would see that almost all the bases coincide, except at specific points on the STI1 and STI2, where marks appear indicating the genetic difference of the Salvadoran species.

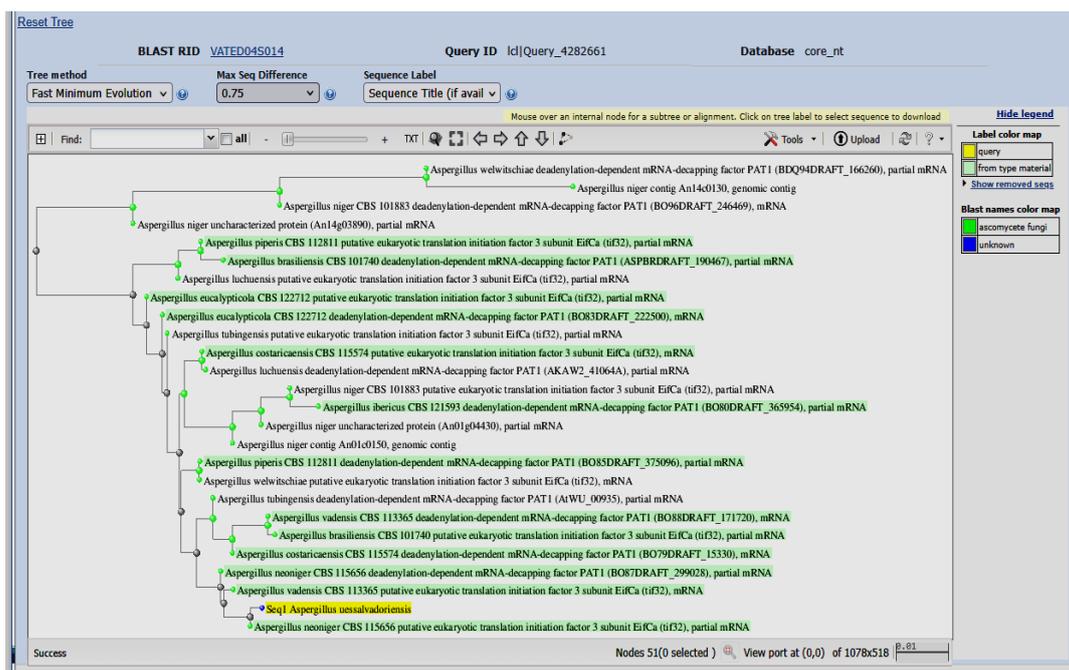


Figure 5. Phylogeny between the species of *Aspergillus* with *uessalvadorensis*. GenBank 2024

In Figure 5 As you can see in the tree, Seq1 *Aspergillus uessalvadorensis* (marked in yellow) appears in a clade (branch) very close to *Aspergillus neoniger* and *Aspergillus vadensis*. This reinforces, although they are close relatives, the tree separates them into different branches, which confirms that they are different species and not the same (there is no specificity).

Conclusion

1. Confirmation of Genomic Identity. The clearest evidence is the length of the branches in the phylogram. In molecular biology, if all three sequences were virtually the same species, we would see extremely short branches or even a single line. However, the values (0.27, 0.20 and 0.29) are robust numerical distances. This confirms that *Aspergillus uessalvadorensis* has a nucleotide signature that does not exactly match either *A. niger* or *A. neoniger*.
2. Resolution of Cryptic Species. The graph shows overcoming the barrier of cryptic species. In the black *Aspergillus* group, many species appear identical under the microscope. The real takeaway here is that the genetic marker you used is sensitive enough to separate *A. neoniger* from *A. niger* and, more importantly, to place your sample in its own independent branch.
3. The Verdict of Distance. If we look at the data objectively, the conclusion is that there is a complex of related but distinct species. The sequence with the distance of 0.29709 is the most divergent of all; has

accumulated almost 10% more changes than the others. If that sequence corresponds to the result, the scientific conclusion is that there is sufficient evolutionary distance to defend its classification as a separate taxonomic entity. In short: There is no ambiguity. The UGENE data says that there is enough genetic noise (accumulated mutations) to conclude that they are three different biological entities. You are seeing the evolution in real time reflected in mathematical distances.

4. Taxonomic and Phylogenetic Conclusion. The phylogenetic analysis carried out by reconstruction by genetic distances allows us to conclude, unequivocally, that there is a significant molecular divergence between the isolates analyzed. The topology of the resulting tree dismisses the specificity between the samples, confirming that the observed differences in nucleotide alignment are not random variations, but indicators of independent evolutionary lineages.

Gratitude and Recognition

To the authorities of the University of El Salvador and the Faculty of Medicine of the UES for their moral support. To the B1 team of MACROGEN, Inc. Biotechnology Company. South Korea.

Conflicts of Interest

The author declares that he has no conflict of interest.

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