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Conservation and Recombinational Contexts in Apoptosis Pathway in *Aspergillus fumigatus*

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Abstract

Introduction: *Aspergillus fumigatus* is a highly pathogenic fungus for immunocompromised patients and invasive aspergillosis exhibits mortality rates around 50-80%. Thus, the apoptosis pathway in this fungus may be an important target for the development of new drugs.

Materials and Methods: From a data bank, we analyzed five different proteins (Bax, Bir, Nma111, Pca1, and Rad9) belonging to the apoptosis pathway in the genome of *Aspergillus fumigatus*. Phylogenetic trees, splits graphs, recombination analyses, and assessment of selection strength from codon to codon were developed to understand the evolution and the function of these proteins in the fungus.

Results: Our results suggest a conservation of these proteins in the Aspergillaceae family, as well as a low rate of gene recombination with a putative preservation of the function.

Conclusion: We thus propose that the apoptosis pathway in *Aspergillus fumigatus* is started with the Rad9 protein followed by the activation of Bax and Pca1 proteins. Also, Nma111 cleaves the Bir protein resulting in the consequent activation of the Bax protein. Further studies are necessary to better understand the apoptosis process in *Aspergillus fumigatus* and to confirm the proteins belonging to the pathway.

Keywords: Apoptosis pathway; *Aspergillus fumigatus*; Bioinformatics; Molecular targets of drugs

Introduction

The genus *Aspergillus* is considered to be ubiquitous and is composed of opportunistic and highly pathogenic organisms. This genus is also responsible for the decomposing plant substrates through diversified and flexible nutritional processes [1]. This genus includes species responsible for human infections such as *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus amstelodami*, *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus fumigatus*, and *Aspergillus sydowi*. Among them, *Aspergillus fumigatus* is the species responsible for the majority of invasive cases of aspergillosis [2,3].

Several studies have been performed using *Aspergillus fumigatus* as a result of the tropism of this species for immunocompromised patients. This invasive tropism is mainly due to its mass production of tiny conidia which may colonize the respiratory tract, reaching the alveoli, which are the main gate for systemic infections triggered by this fungus [4,5]. This high ability to become infectious may cause mortality in about 50-80% of the cases [6]. Apoptosis is a natural process occurring in yeast and it is well characterized in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* [7], although it has not been described yet in *Aspergillus fumigatus*. This process is involved in cellular regulation and maintenance and may occur through various external and internal stimuli [8]. Pro-apoptotic proteins (Bax and Bak) belonging to the Bcl-2 family are transferred from the cytosol to the mitochondria in a movement that releases pro-apoptotic mediators such as cytochrome C, Ca^{2+}, and inhibitors of apoptosis proteins (IAPs). Bax makes a complex with cytochrome C and binds to procaspase 9, activating the caspase cascade [9-11].

In fungi, instead of caspases, there is an involvement of metacaspases in the apoptotic process, which are proteins belonging to a distant family of caspases. Despite having a similar structure, the metacaspases lack aspartate specificity, cleaving their targets after lysine and arginine residues. The metacaspases play important roles in the apoptosis process. The fungus *Aspergillus fumigatus* has two metacaspases, named CasA and CasB, and the deletion of both of them undermines its growth under endoplasmic reticulum stress [12]. The searching for new effective therapeutic targets specific to *Aspergillus fumigatus* is greatly demanded. Due to the failure of the available therapies, it is urgent to study the signaling pathways present in this microorganism, in order to discover molecular targets for the development of new drugs. The apoptotic process, however, is also directly related to a complex network of signaling proteins [13].

Therefore, this work aimed to review the conservation of five validated proteins (Bax, Bir, Nam111, Pca1, and Rad9) which are involved in the apoptosis pathway of this fungus, comparing their recombination patterns to generate an interaction network and an evolutionary approach to determine the cascade of apoptosis for *Aspergillus fumigatus* and reinforce its use for control.

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Materials and Methods

Data mining and biological sequences

The analyzed proteins were chosen after an extensive research in the literature, and the sequences were retrieved from the database of the ortholog *Schizosaccharomyces pombe* (http://www.pombase.org/). These proteins were then searched in the genome of the fungus *Aspergillus fumigatus* (www.aspgd.org/) and also in ortholog organisms using the BLASTp tool. Only protein sequences whose identity was higher than 70% (e value $<10^{-5}$) were used. The gene and mRNA sequences that codify these proteins were retrieved from the NCBI site (https://www.ncbi.nlm.nih.gov/) (Table S1) and the Conserved Domains Database (CDD) was consulted for the analysis of domains.

Phylogenetic reconstruction

The evolutionary analysis of *Aspergillus fumigatus* was carried out using MEGA 5 software [14]. Phylogenetic trees were constructed based on the amino acid sequences for all the tested genes in order to eliminate codon usage bias. Protein sequences were obtained from online open databases as previously referred (Table S1), and were MUSCLE aligned and trimmed with the g-Block algorithm (http://phylogeny.lirmm.fr/phylo_cgi/one_task.cgi?task_type=gblocks) in PhyML 3.0 server (http://phylogeny.lirmm.fr/phylo_cgi/one_task.cgi?task_type=phyml). A substitution model for each data set was obtained through the internal algorithm implemented in the MEGA software and was determined with the aid of the Akaike Information Criterion (AIC). Trees were constructed using the Maximum Likelihood (ML) algorithm from initial trees obtained using the Nearest-Neighbor Interchange (NNI)/BioNeighbor-Joining (BioNJ) algorithm corrected using the best substitution model. Node support was evaluated through bootstrapping of 1000 pseudo-replicates (Table S2).

Analysis of reticulation

Splits graphs for trend evaluation of reticulate evolution were obtained from the conversion of alignment data previously obtained by the software SplitsTree4-Version 4.13.1 [15]. The positions containing gaps and parsimony from uninformative sites were deleted in order to build the final chart. The final tree had its nodes tested using the bootstrap approach with 1000 pseudo-replicates.

Extension of recombination events

The determination of the extent of recombination was performed using different approaches. The GARD tool, available at the Datamonkey server (http://www.datamonkey.org/), was used to evidence phylogenetic inconsistencies and to identify the number and location of breaking points corresponding to recombination events. The validation of results was carried out with eight different methods implemented in the Recombination Detection Program v.4.0 (RDP): RDP [16], GENECONV [17], Bootscan [18], Chimera [19], SiScan [20], LARD [21], MaxChi [22], and 3Seq [23]. The analysis was performed with default settings for detection methods with Bonferroni corrected parameter for a P-value of 0.05, and a requirement that every event must be detected simultaneously by four or more methods. The mRNA data were also used to evaluate the effects of different codon usage.

Results and Discussion

Bax

The best amino acids substitution model was Dayhoff+G+F (BIC of 11.133.635, AIC of 10.577.339, lnL of -5.206.745, Invariant sites n/a and Gamma parameters of 0.77). Final tree configuration shows that members of the family Aspergillaceae are held together displaying the conservation of proteins within the family (Figure 1).

In Figure 1, the organisms were divided into five groups. Group I is a monophyletic group, in which all fungi are found in most environments and are important plant pathogens or appear in association with algae to form lichens. Group II consists of filamentous fungal pathogens of humans. Members of the families Aspergillaceae and Trichocomaceae are present in the same clade. Groups III, IV and V consisted of singletons of higher eukaryotes.

Bax and Bak are pro-apoptotic proteins which are effectively activated when their C-terminal conformation is changed to interact with a BH3 domain of the Rad9 protein. This direct interaction is more likely to occur compared to the binding of the Rad9 protein, Bcl-2/Bcl-xL and the subsequent binding of Bax/Bak proteins. Instead of Bax/ Bak activation, the formation of pores in the outer mitochondrial membrane and the release of the cytochrome C take place [24].

The figure shows the phylogenetic tree of the entire recovered sequences. It is noted that the family Aspergillaceae remains a monophyletic group with a bootstrap of 95%. This tree shows a topological incongruence which is a consequence of the difference in the full protein sequence. That difference can be due to the substitution or insertion of amino acids in the polypeptide chain, which highlights the importance of the domain analysis. From the protein domain analysis (Table 1) it was possible to confirm the presence of domains that are directly related to the apoptotic process. The GAAP domain present in *Aspergillus fumigatus* is involved in the apoptosis regulation. This domain is related to the superfamily (BI) -1 like and presents apoptotic and anti-apoptotic function when the stimulation of the Bcl-2 oncogene occurs or when the inhibition of the effect of Bax takes place [25]. The Bax protein has a conserved function like pro-apoptotic proteins in all organisms [26-28]. *Drosophila ananassae*, however, presents one different domain with opposite functions. The LFG domain appears in the place of GAAP. The first is a putative regulator of apoptosis activation, while the second acts inhibiting or activating the apoptosis process [29]. The loss of the apoptosis activation function can be responsible for the location and organization of an organism in the phylogenetic tree. The arrangement of the GAAP and LFG domains in the protein may be related to the activation of the apoptosis function, since in both organisms the domains occupy almost all the extension of the protein. The GAAP domain is conserved in higher organisms, evolutionarily distant from fungi, such as *Arabidopsis thaliana* and *Homo sapiens.* This suggests that despite the evolution of organisms, the function of the domain remains conserved, proving to be important for the activation of apoptosis. The absence of the activation of the apoptosis function in the LFG domain in *Drosophila ananassae* may be related to the lack of a protein similar to the BH3 protein [30].

Another factor influencing the organization of organisms in the phylogenetic tree is the genetic recombination. This process promotes the formation of new genes from the combination of genes from different organisms, allowing the addition or loss of a function. However, the network splits graph (Figure 2) obtained for Bax protein shows no recombinations, corroborating the results found in RDP. Besides, Figure 2 showed the longer arms of a network consisting of *Homo sapiens*, *Drosophila ananassae,* and *Arabidopsis thaliana*, which are evolutionarily distant from the other clusters. The Aspergillaceae family clade stands clearly separated from other clades which show protein conservation, confirming the results obtained at the phylogenetic reconstruction. Although not presenting recombination,

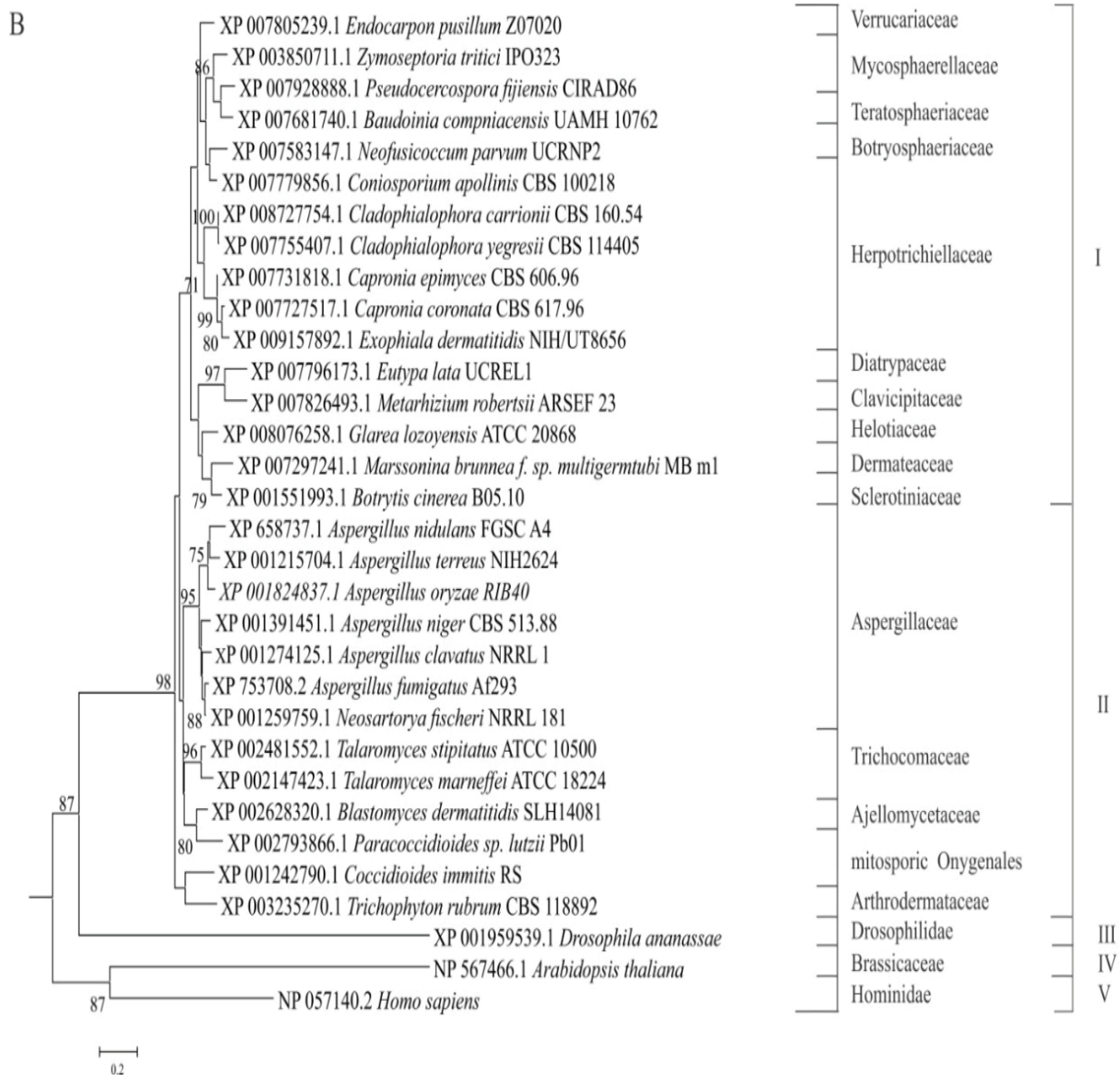

Figure 1: Molecular Phylogenetic Analysis by Maximum Likelihood Method. The evolutionary history of Bax using complete Bax protein (A) and G-block cured Bax protein (B). All positions containing gaps and missing data were eliminated.

Organism	Domain	Accession	Interval
Aspergillus fumigatus	GAAP_like	cd10429	36-268
	Bax1-I	pfam01027	64-264
Drosophila ananassae	LFG_like	cd10428	26-243
	Bax1-I	pfam01027	36-242

Table 1: Domains of Bax protein. Domains were obtained from analyses of the Conserved Domain Database (CDD). Table refers to the organism under study, the name of the domain, the access code of the domain and the size of the domain. The table compares domains of Bax protein from other organisms to domains of Bax protein in *Aspergillus fumigatus*.

Figure 2 describes an evolutionary history, where the nodes represent the ancestors and the edges the descent patterns. Recombination analysis for Bax did not result in possible recombinations.

Trends undergoing positive selection were not confirmed by the selection server (Figure S1). Conversely, Figure S1 shows that proteins undergo negative selection, suggesting a limitation in the amino acid sequence change, upholding very important structures in order to maintain the protein function.

Bir

In this analysis, WAG+G+I was used as the amino acid substitution model (BIC of 2991.96, AIC of 2679.669, lnL of -1278.521, Invariant sites of 0.13 and Gamma parameters of 1.6), which generated a phylogenetic tree displayed in Figure 3. By observing the phylogenetic tree for Bir protein, it is possible to note a distribution rate in many clusters suggesting a conservation pattern for this protein. The Aspergillaceae family belongs to Group I and is represented by a monophyletic group which forms a single clade. Group II consists of the cluster of

Organism	Domain	Accession	Interval
Aspergillus fumigatus	BIR	cd00022	5-91
	BIR	cd00022	117-186
	BIR	pfam00653	7-93
	BIR	pfam00653	119-185
	BIR	smart00238	3-92
	BIR	smart00238	117-185
Drosophila melanogaster	BIR	cd00022	29-102
	BIR	pfam00653	31-101
	BIR	smart00238	27-102
Homo sapiens	CARD_BIRC2_BIRC3	cd08329	441-530
	BIR	cd00022	256-322
	BIR	cd00022	171-237
	BIR	cd00022	32-98
	RING	cd00162	556-595
	BIR	smart00238	254-322
	BIR	smart00238	168-237
	BIR	smart00238	28-98
	BIR	pfam00653	258-323
	BIR	pfam00653	172-236
	BIR	pfam00653	32-97
	CARD	smart00114	441-527
	CARD	pfam00619	446-528
	UBA_BIRC2_3	cd14394	377-411
	zf-C3HC4_3	pfam13920	553-598
	RING	smart00184	557-591

Table 2: Domains of Bir protein. Domains were obtained from analyses of the Conserved Domain Database (CDD). Table refers to the organism under study, the name of the domain, the access code of the domain and the size of the domain. The table compares domains of Bir protein from other organisms to domains of Bir protein in *Aspergillus fumigatus*.

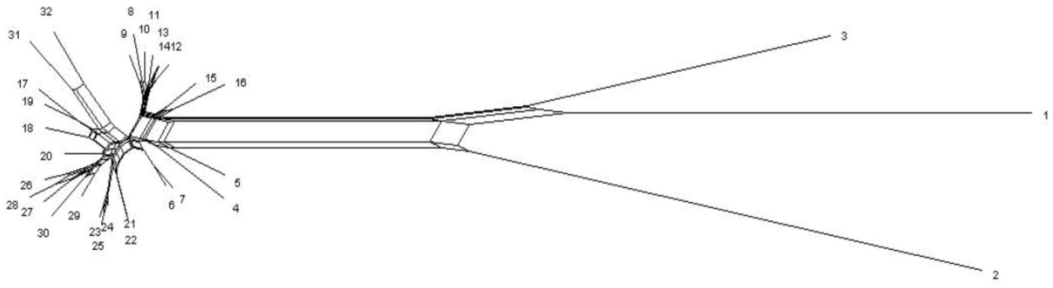

Figure 2: Splits graph of Bax protein. The uninformative parsimony sites and gaps were excluded. One thousand (1000) pseudo replications were made to evaluate node support. Final tree was corrected using ProteinML model. The homologous organisms are represented**:** 1-*Arabidopsis thaliana*, 2-*Drosophila ananassae*, 3-*Homo sapiens*, 4-*Trichophyton rubrum* CBS118892, 5-*Coccidioides immitis RS*, 6-*Talaromyces stipitatus* ATCC10500, 7-*Talaromyces marneffei* ATCC18224, 8-*Aspergillus nidulans* FGSCA4, 9-*Aspergillus terreus* NIH2624, 10-*Aspergillus oryzae* RIB40, 11-*Aspergillus niger* CBS513.88, 12-*Aspergillus clavatus* NRRL1, 13- *Neosartorya fischeri* NRRL181, 14-*Aspergillus fumigatus* Af293, 15-*Blastomyces dermatitidis* SLH14081, 16-*Paracoccidioides sp. lutzii* Pb01, 17-*Marssonina brunnea f. sp. multigermtubi* MBm1, 18-*Glarea lozoyensis* ATCC20868, 19-*Botrytis cinereal* B05.10, 20-*Endocarpon pusillum* Z07020, 21-*Cladophialophora carrionii* CBS160.54, 22-*Cladophialophora yegresii* CBS114405, 23-*Exophiala dermatitidis* NIH/UT8656, 24- *Capronia epimyces* CBS606.96, 25-*Capronia coronate* CBS617.96, 26-*Baudoinia compniacensis* UAMH10762, 27-*Zymoseptoria tritici* IPO323, 28-*Pseudocercospora fijiensis* CIRAD86, 29-*Coniosporium apollinis* CBS100218, 30-*Neofusicoccum parvum* UCRNP2, 31-*Eutypa lata* UCREL1, 32-*Metarhizium robertsii* ARSEF23.

Eurotiomycetes. This class is formed by a monophyletic group and two clades comprising Eurotiomycetidae and Chaetothyriomycetidae subclasses [30]. In this tree under study, three subclasses were separated, although the organism has not originated a monophyletic group. *Glarea lozoyensis*, as well as other Group II members, are found in water and small plants but is not part of the class Eurotiomycetes. The remaining organisms were grouped according to their class.

In Figure 3, the presence of incongruences shows changes in the full sequence of the protein. The organisms in the class Eurotiomycetes behaved differently from the previous tree. In this case, they set new descendants forming new clades. The Eurotiales class, which belongs to the family Aspergillaceae, was very similar in both trees, suggesting that the complete sequence of these organisms has not undergone many divergences. The BIR domain is conserved among the organisms, and this can also be verified in *Homo sapiens*, *Drosophila melanogaster* and *Aspergillus fumigatus* (Table 1). These organisms are evolutionarily distant but exhibit BIR superfamilies in addition to zinc binding sites. The BIR domain is related to the inhibition of apoptosis in humans via caspases. The Bir protein, with its anti-apoptotic function, acts as a substrate for the Nma111 protein. Bir is directly degraded by Nma111 and presents no direct link to the metacaspase Pca1. The degradation

of Bir antagonizes the inhibitory effect and promotes apoptosis [31].

By analyzing the reticulated network of the Bir protein (Figure 4) it is clear that there is a complex relationship between the organisms, suggesting the presence of reticulate events such as hybridization, horizontal gene transfer, or recombination. These events are represented by the edges, and the nodes are the hypothetical ancestors resulting from reticulate events. Although SplisTree suggests the presence of recombinant events, RDP analysis did not result in recombination rates that were within our standard of analysis.

The tendency to experience positive selection may help to understand the differences found among the organisms. The Tajima's statistical test showed a value of 2.780379 suggesting a positive selection. The selecton server (Figure S2) was used to confirm this data and the result suggests that the selection does not occur in a diversifying way. This demonstrates that the structure and/or function of the protein do not allow changes.

Nma111

Phylogenetic reconstructions were based on corrections made using the WAG+G model of amino acid substitution (BIC of 22.237.401, AIC of 21.779.377, lnL of -10.827.363, Invariant sites of n/a and Gamma parameters of 1.38). The phylogenetic tree obtained can be seen in Figure 5. Group I consists of organisms that belong to the class Eurotiomycetes and are distributed among the subclasses Eurotiomycetida and Chaetothyriomycetida. The family Aspergillaceae forms a monophyletic group and has very short arms, which suggest that mutation rates for the Nma111 protein are short.

Domains indirectly linked to apoptosis were found in the Nma111 protein from *Aspergillus fumigatus* (Table 1). The PDZ serine protease domain is directly related to apoptosis and is superimposed on the domain degP htrA DO. This domain mediates and also promotes the cleavage of the Bir protein as a consequence of its pro-apoptotic function. Domains such as PDZ 1 are shared among bacteria, fungi, and plants [32]. In *Homo sapiens,* the superfamily Kazal was found, which is formed by protease inhibitors [33], in addition to the degP htrA DO domain. The presence of the superfamily Kazal could explain the evolutionary distance of *H. sapiens* to others and suggests a gain of function by the protein after evolutionary processes. The arrangement of the organisms in the phylogenetic tree can be compared to the splits network (Figure 6) in which the length of an edge is proportional to the length of the arms on the phylogenetic tree. The splits graphs show long arms to cluster the operation taxonomic units of *Homo sapiens* and *Drosophila persimilis*. The presence of reticulate events was observed neither in the splits graph nor in the recombination analysis with RDP, which did not show any significant recombination events.

Analysis using the selecton server (Figure S3) did not reveal any significant trend to a positive selection in any protein coding segments. This result suggests that the structure and/or function of the protein is preserved.

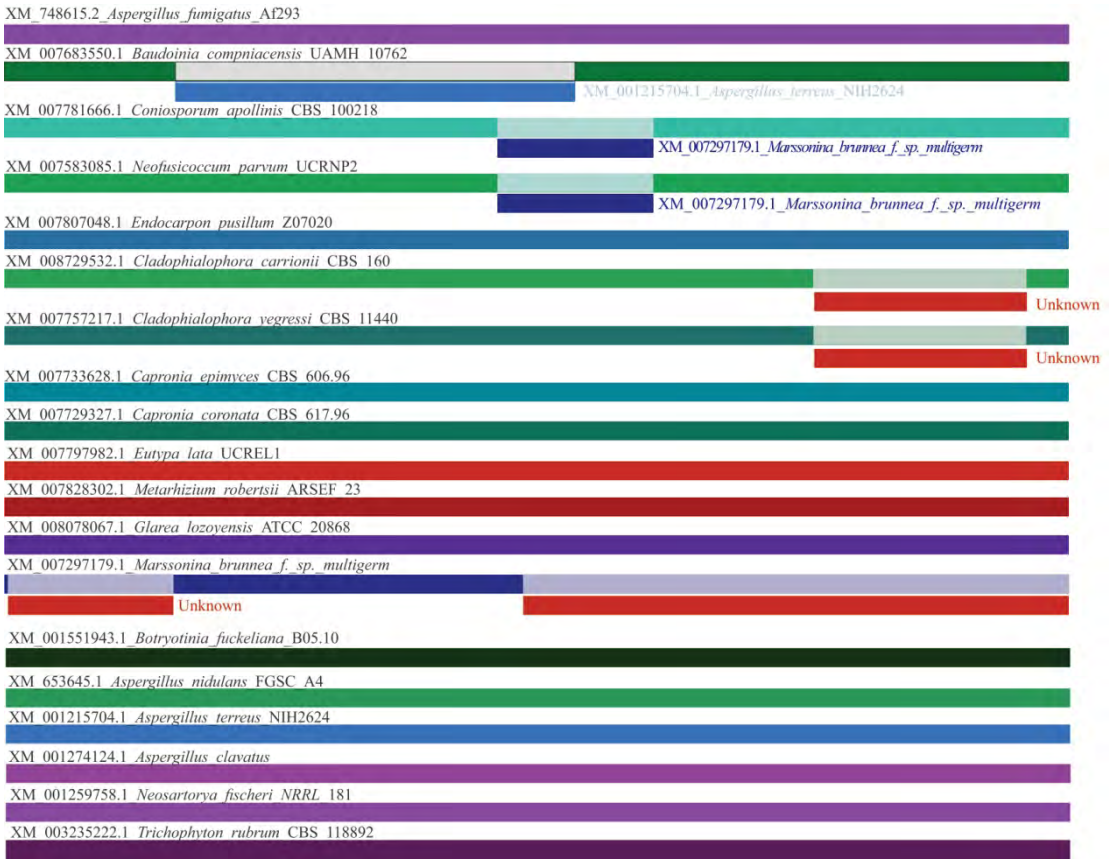

Figure 3: Recombination events involved in Bax evolution. Each sequence is represented by a color and the recombination is evidenced by the donor.

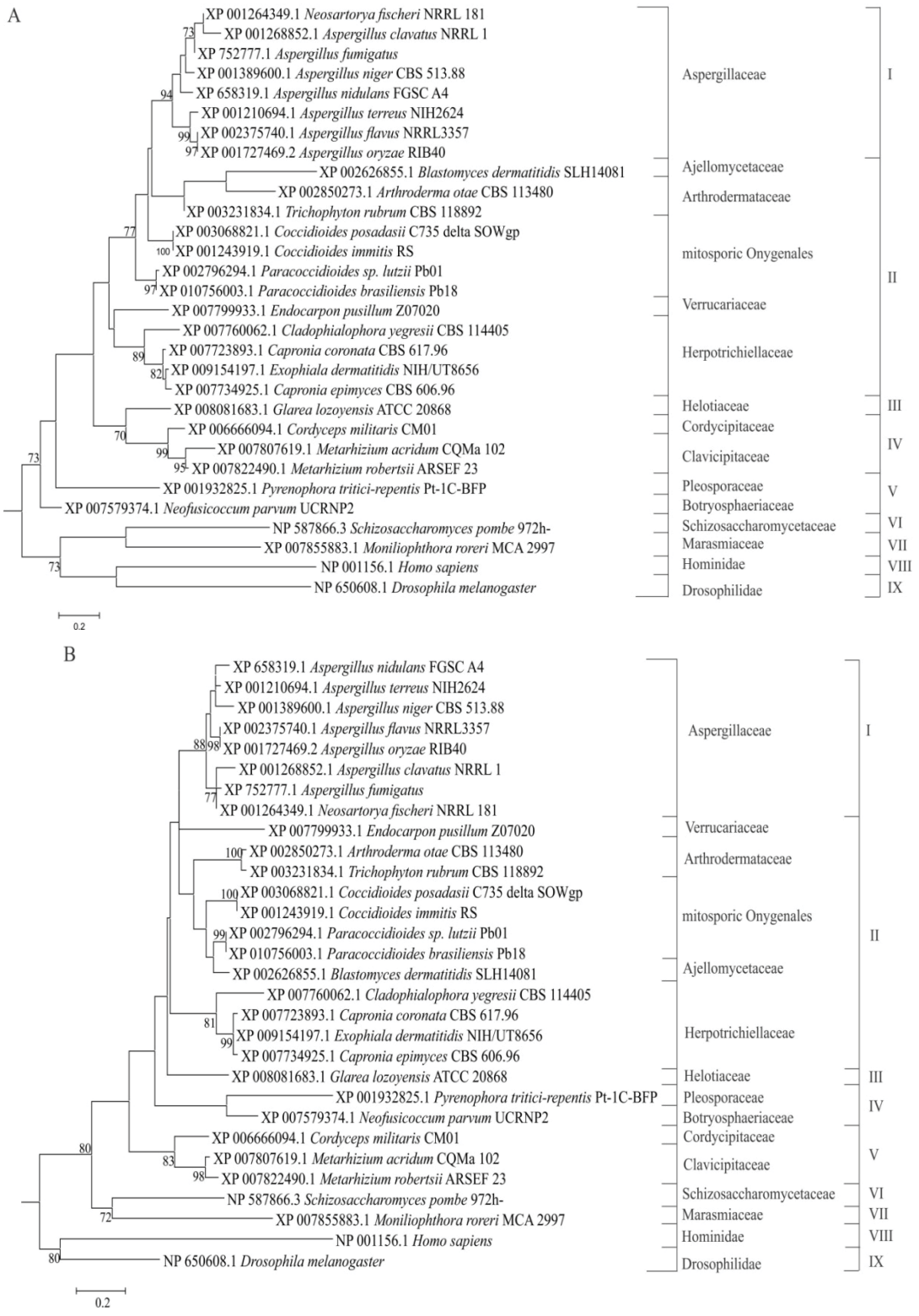

Figure 4: Molecular phylogenetic analysis by Maximum Likelihood (ML) method. The evolutionary history of Bir using complete Bir protein (A) and G-block cured Bir protein (B). All positions containing gaps and missing data were eliminated.

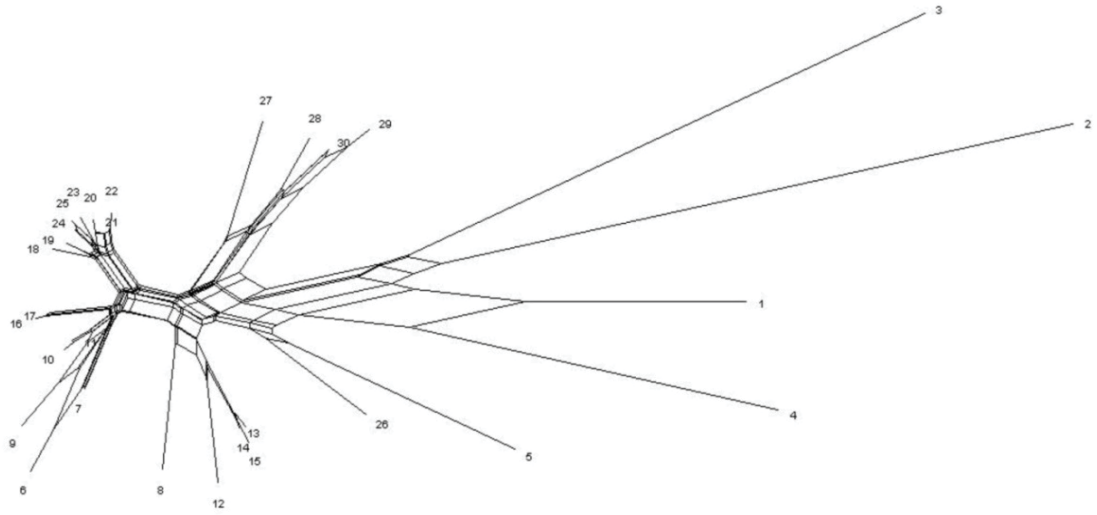

Figure 5: Splits graph of Bir protein. The uninformative sites of thrift, gaps and sites listed were excluded. One thousand (1000) pseudo replications were made to sustain the high reliability. We used the model ProteinML correction. The homologous organisms are represented: 1-*Schizosaccharomyces pombe* 972h, 2-*Homo sapiens*, 3-*Drosophila melanogaster*, 4-*Moniliophthora roreri* MCA 2997, 5-*Pyrenophora tritici-repentis* Pt-1C-BFP, 6- *Arthroderma otae* CBS113480, 7-*Trichophyton rubrum* CBS118892, 8-*Endocarpon pusillum* Z07020, 9- *Blastomyces dermatitidis* SLH14081, 10-*Paracoccidioides sp. Lutzii* Pb01, 11-*Paracoccidioides brasiliensis* Pb18, 12-*Cladophialophora yegresii* CBS114405, 13-*Capronia coronata* CBS 617.96, 14- *Exophiala dermatitidis* NIH/UT8656, 15- *Capronia epimyces* CBS606.96, 16- *Coccidioides posadasii* C735 delta SOWgp, 17-*Coccidioides immitis* RS, 18-*Aspergillus nidulans* FGSC A4, 19-*Aspergillus niger* CBS513.88, 20- *Aspergillus flavus* NRRL3357, 21-*Aspergillus oryzae* RIB40, 22-*Aspergillus terreus* NIH2624, 23-*Aspergillus clavatus* NRRL1, 24-*Aspergillus fumigatus*, 25-*Neosartorya fischeri* NRRL181, 26-*Neofusicoccum parvum* UCRNP2, 27-*Glarea lozoyensis* ATCC 20868, 28-*Cordyceps militaris* CM01, 29-*Metarhizium acridum* CQMa 102, 30-*Metarhizium robertsii* ARSEF 23.

Pca1

The phylogenetic tree for this protein was made using the WAG+G model of amino acid substitution based on the parameters BIC of 9151.580978, AIC of 8684.844288, lnL of -4275.918097, Invariant sites of n/a and Gamma parameters of 0.545140.

The main operational taxonomic units could be clustered at least in four different groups. Group I is represented by filamentous fungi that are ubiquitous pathogens. In this group the family Aspergillaceae remained in a monophyletic group, suggesting a conserved evolution in the family organization. Group II is represented by fungi found in plants, many of which are important grain pathogens. Group III contains yeasts, which are evolutionarily distant and do not share the same common ancestor. Group IV is represented by *Arabidopsis thaliana*. In Figure 7, the arms are longer, suggesting a higher level of evolutionary changes.

The yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* form the outgroup of *Arabidopsis thaliana*. The arrangement of the groups in the phylogenetic tree suggests the presence of recombination events (Tables 1-5) even though the peptidase C14 domain is conserved among organisms. This domain is a caspase domain, related to cell death. In other higher eukaryotes, cytochrome C is responsible for the activation of the caspase pathway. It is known that fungi do not present caspases, but metacaspases instead. The metacaspase Pca1 was suggested to participate in the apoptosis, although its real participation in the pathway is not well defined [34]. Analysis of the splits graph (Figure 8) indicates the existence of possible genetic recombination between the organisms. However, this result was not confirmed by the RDP. Recombinant events were not within the proposed statistics for the article.

The recombinant events in the splitstree suggest a high rate of horizontal transfer suggesting hybridization or recombination. The long arms of the splitstree are composed of evolutionarily distant organisms like *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, and *Arabidopsis thaliana*. Although there are two fungi segregated as an outgroup, both suffered little recombination, being separated from the other fungi.

The high rate of recombination can explain the differences between the organisms, besides the tendency to undergo positive selection, which also contributes to a better understanding of such differences. The value obtained in the Tajima statistical test was -0.284318 indicating a tendency for negative selection. This result was confirmed by the analysis of the selecton server (Figure S4) that indicates that the negative selection in function and/or protein structure is important and do not accept changes, suggesting a conservation of the protein between organisms.

Rad9

The phylogenetic tree for this protein was made using the JTT+G model of amino acid substitution based on the parameters BIC of 8654.559155, AIC of 8308.933614, lnL of -4097.579401, Invariant sites of n/a and Gamma parameters of 2.074206.

In Figure 9, the phylogenetic tree shows a clear clustering trend to form three main groups. Group I consists of filamentous fungi and pathogens with the family Aspergillaceae behaving like a monophyletic group. Group II consists of fungi present in plants that are evolutionarily distant when compared to Group I.

The Rad9 protein is a component of the BH3-only family which has pro-apoptotic function and is required for the apoptosis

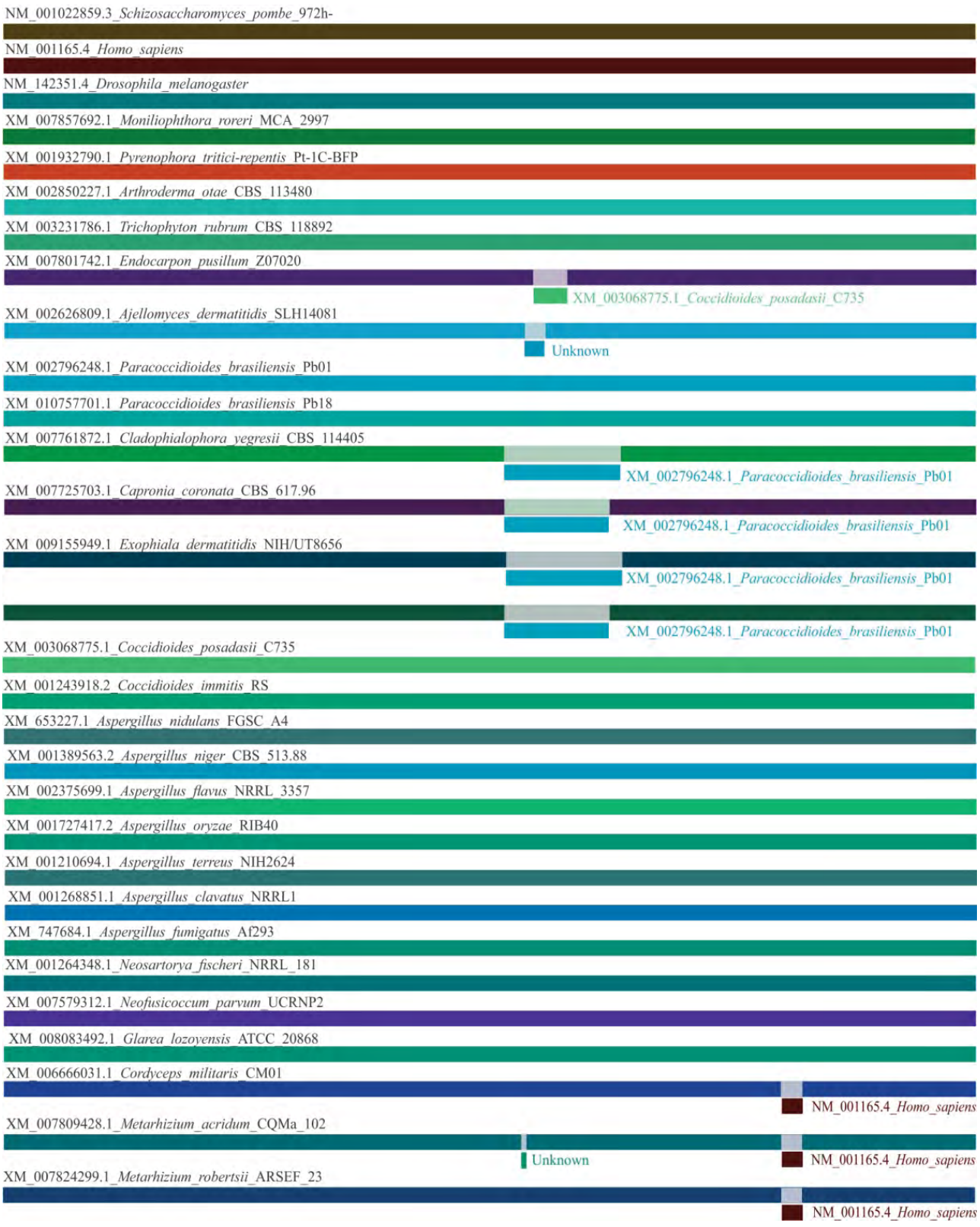

Figure 6: Recombination events involved to Bir evolution. Each sequence is represented by a color and the recombination is evidenced by the donor.

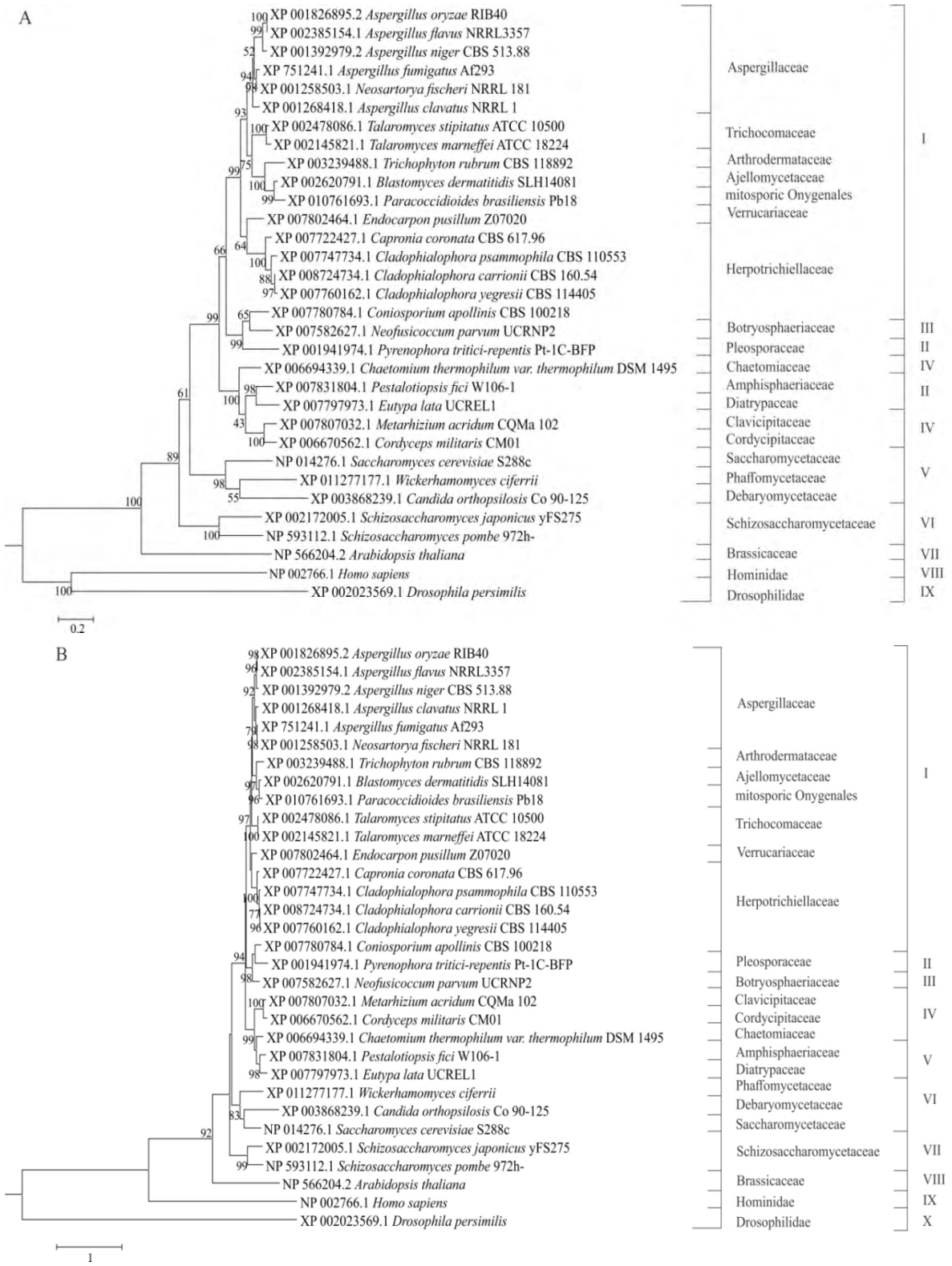

Figure 7: Molecular phylogenetic analysis by Maximum Likelihood (ML) method. The evolutionary history of Nma111 using complete Nma111 protein (A) and G-block cured Nma111 protein (B). All positions containing gaps and missing data were eliminated.

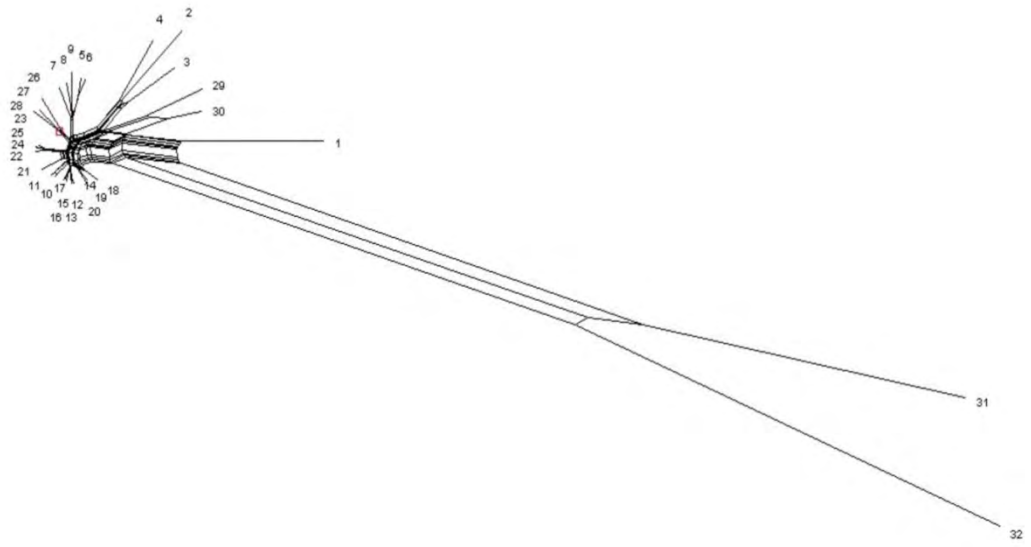

Figure 8: Splits graph of Nma111 protein. The uninformative sites of thrift, gaps and sites listed were excluded. One thousand (1000) pseudo replications were made to sustain the high reliability. We used the model ProteinML correction. The homologous organisms are represented: 1-*Arabidopsis thaliana,* 2-*Candida orthopsilosis,* 3-*Wickerhamomyces ciferrii,* 4-*Saccharomyces cerevisiae,* 5-*Metarhizium acridum,* 6-*Cordyceps militaris,* 7-*Chaetomium thermophilum var. thermophilum,* 8-*Pestalotiopsis fici,* 9-*Eutypa lata,* 10-*Talaromyces stipitatus,* 11-*Talaromyces marneffei,* 12-*Aspergillus niger,* 13-*Aspergillus oryzae,* 14-*Aspergillus flavus,* 15- *Aspergillus clavatus,* 16-*Aspergillus fumigatus,* 17-*Neosartorya fischeri,* 18-*Trichophyton rubrum,* 19- *Blastomyces dermatitidis,* 20-*Paracoccidioides brasiliensis,* 21-*Endocarpon pusillum,* 22-*Capronia coronata,* 23-*Cladophialophora psammophila,* 24-*Cladophialophora carrionii,* 25-*Cladophialophora yegresii,* 26-*Pyrenophora_tritici repentis,* 27-*Coniosporium apollinis,* 28-*Neofusicoccum parvum,* 29-*Schizosaccharomyces japonicus,* 30-*Schizosaccharomyces pombe,* 31-*Homo sapiens,* 32-*Drosophila persimilis*.

Organism	Domain	Accession	Interval
Aspergillus fumigatus	PDZ_serine_protease	cd00987	299-384
	PDZ_1	pfam12812	391-467
	PDZ_1	pfam12812	868-946
	PDZ_2	pfam13180	327-386
	PDZ_serine_protease	cd00987	900-941
	PDZ	smart00228	327-377
	PDZ_serine_protease	cd00987	817-861
Arabidopsis thaliana	PDZ_serine_protease	cd00987	280-366
	PDZ_serine_protease	cd00987	985-1067
	PDZ_1	pfam12812	373-446
	PDZ_2	pfam13180	305-368
	PDZ_1	pfam12812	971-1047
	PDZ	smart00228	304-359
	PDZ_serine_protease	cd00987	893-961
Homo sapiens	PDZ_serine_protease	cd00987	382-473
	KAZAL_FS	cd00104	114-155
	PDZ	smart00228	403-466
	PDZ_2	pfam13180	410-473
	KAZAL	smart00280	115-155
	Kazal_2	pfam07648	117-155

Table 3: Domains of Nma111 protein. Domains were obtained from analyses of the Conserved Domain Database (CDD). Table refers to the organism under study, the name of the domain, the access code of the domain and the size of the domain. The table compares domains of Nma111 protein from other organisms to domains of Nma111 protein in *Aspergillus fumigatus*.

Organism	Domain	Accession	Interval
Aspergillus fumigatus	Peptidase_C14	pfam00656	206-496
	COG4249	COG4249	204-358
Saccharomyces cerevisiae	Peptidase_C14	pfam00656	136-429
	COG4249	COG4249	134-278
	PRK10263	PRK10263	72-126
Schizosaccharomyces pombe	Peptidase_C14	pfam00656	130-418
Arabidopsis thaliana	Peptidase_C14	pfam00656	80-316
	zf-LSD1	pfam06943	18-42

Table 4: Domains of Pca1 protein. Domains were obtained from analyzes of the Conserved Domain Database (CDD). Table refers to the organism under study, the name of the domain, the access code of the domain and the size of the domain. The table compares domains of Pca1 protein from other organisms to domains of Pca1 protein in *Aspergillus fumigatus*.

Organism	Domain	Accession	Interval
Aspergillus fumigatus	PCNA	cd00577	34-193
	Rad9	pfam04139	13-195
Arabidopsis thaliana	PCNA	cd00577	11-253
	Rad9	pfam04139	13-299
Schizosaccharomyces octosporus	PCNA	cd00577	8-301
	Rad9	pfam04139	13-300
Schizosaccharomyces pombe	PCNA	cd00577	9-301
	Rad9	pfam04139	13-300
Drosophila melanogaster	PCNA	cd00577	7-262
	Rad9	pfam04139	13-258
Homo sapiens	PCNA	cd00577	9-267
	Rad9	pfam04139	13-265

Table 5: Domains of Pca1 protein. Domains were obtained from analyses of the conserved domain database (CDD). Table refers to the organism under study, the name of the domain, the access code of the domain and the size of the domain. The table compares domains of Rad9 protein from other organisms to domains of Rad9 protein in *Aspergillus fumigatus*.

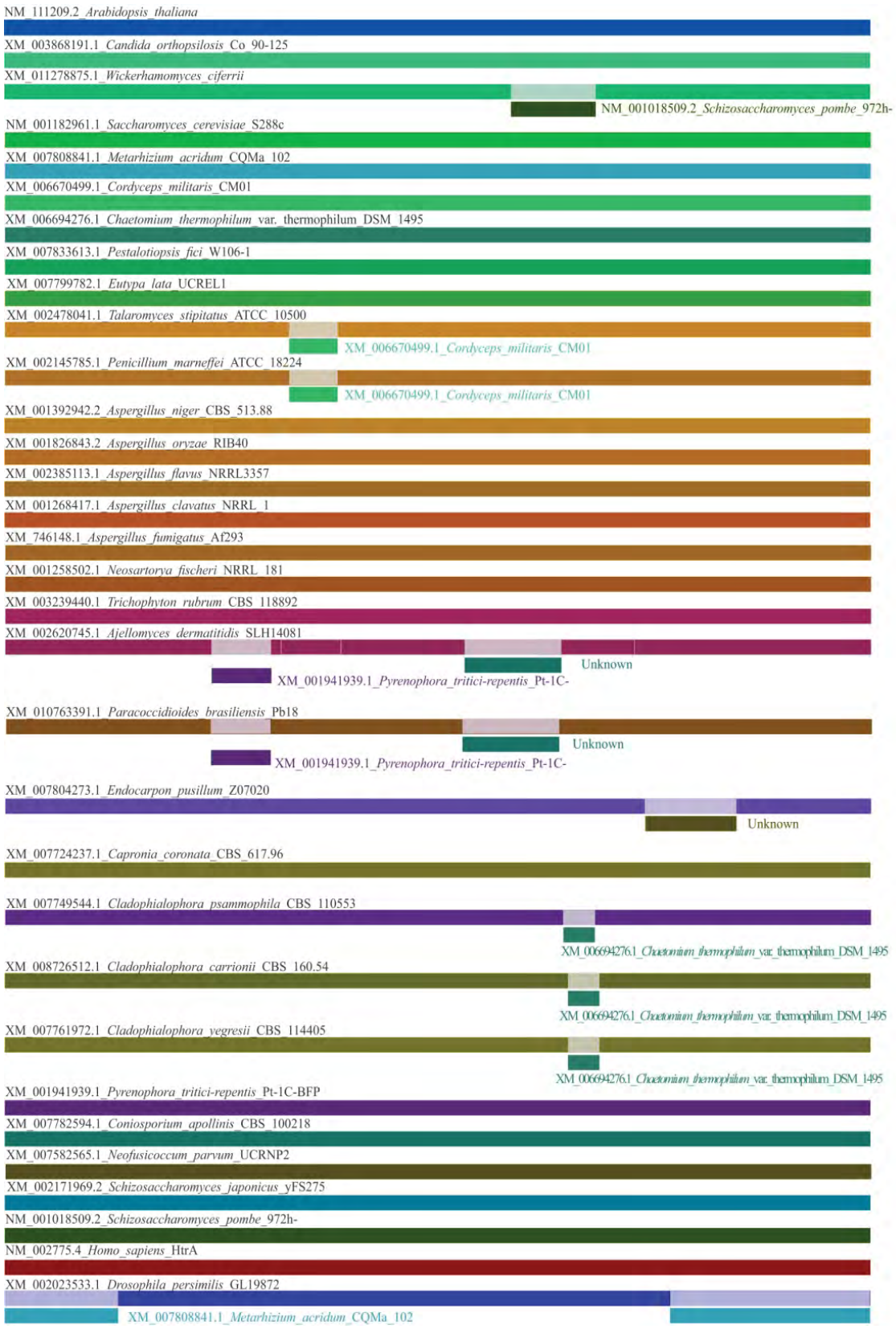

Figure 9: Recombination events involved to Nma111 evolution. Each sequence is represented by a color and the recombination is evidenced by the donor.

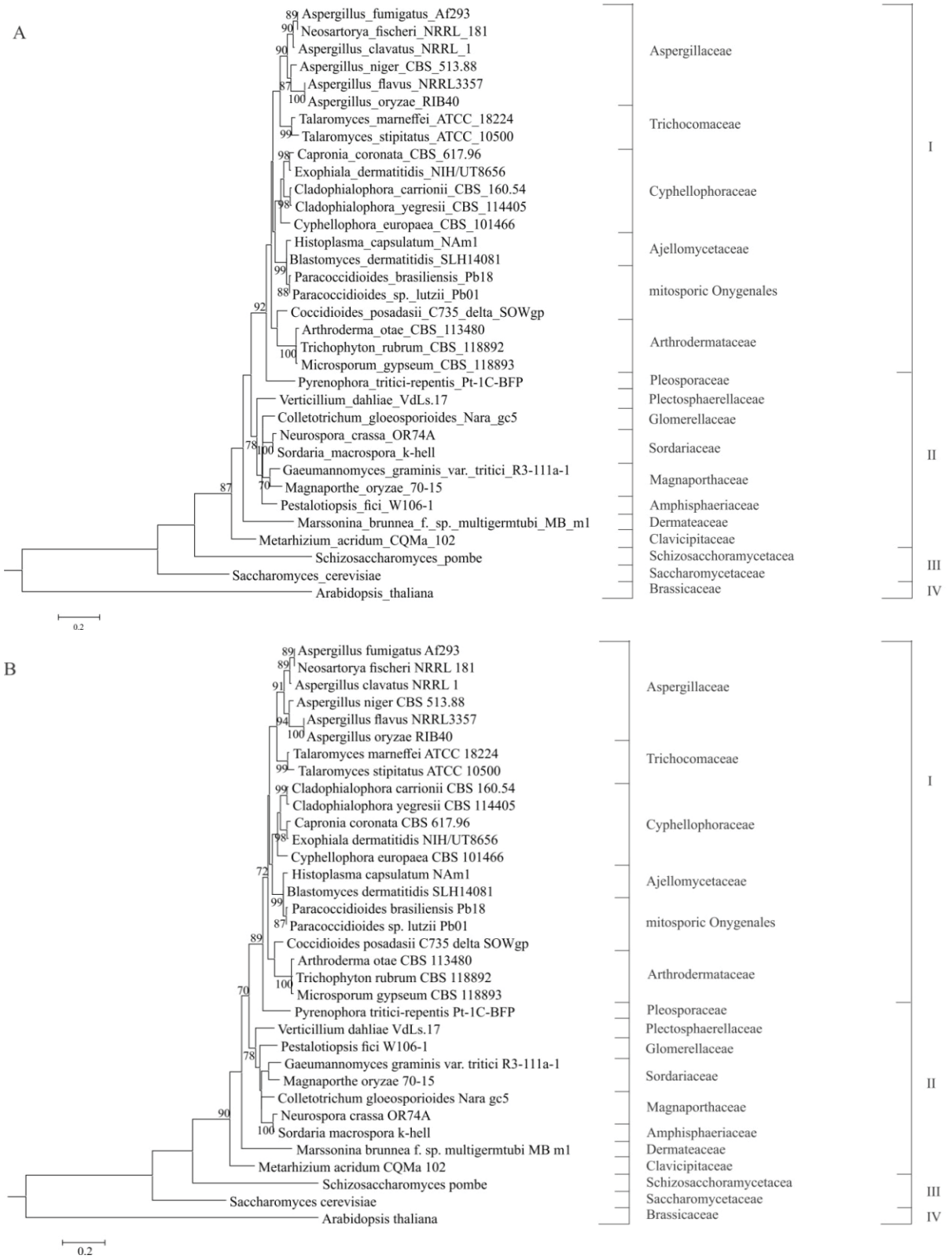

Figure 10: Molecular phylogenetic analysis by Maximum Likelihood (ML) method. The evolutionary history of Pca1 using complete Pca1 protein (A) and G-block cured Pca1 protein (B). All positions containing gaps and missing data were eliminated.

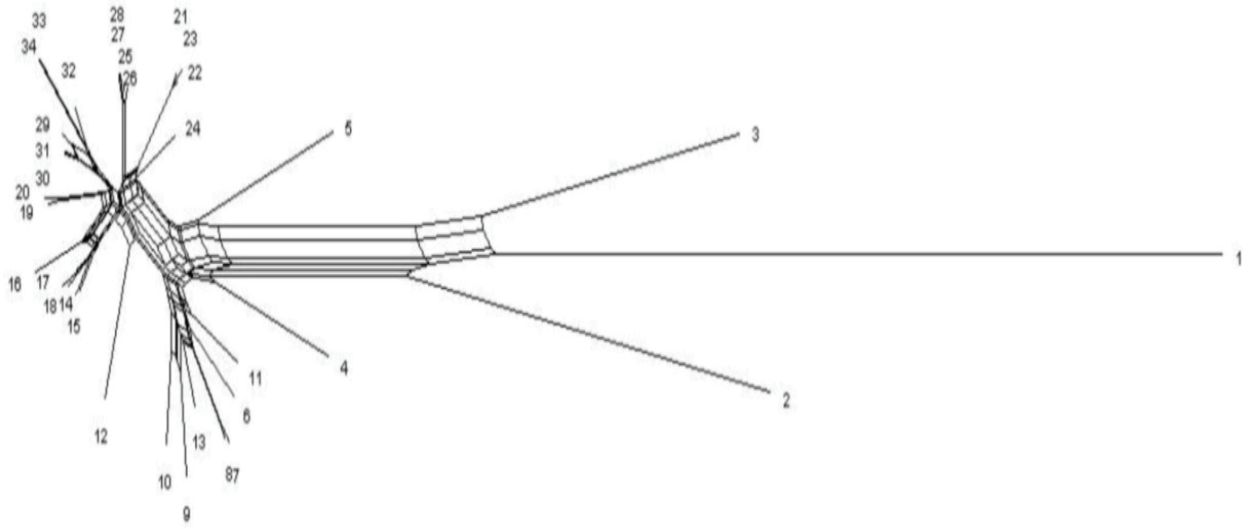

Figure 11: Splits graph of Pca1 protein. The uninformative sites of thrift, gaps and sites listed were excluded. One thousand (1000) pseudo replications were made to sustain the high reliability. We used the model ProteinML correction. The homologous organisms are represented: 1-*Arabidopsis thaliana*, 2- *Schizosaccharomyces pombe*, 3-*Saccharomyces cerevisiae*, 4-*Metarhizium acridum* CQMa102, 5-*Marssonina brunnea f. sp. Multigermtubi* MBm1, 6-*Pestalotiopsis fici* W106-1, 7-*Neurospora crassa* OR74A, 8-*Sordaria macrospora_k-hell*, 9-*Gaeumannomyces graminis var. tritici* R3-111a-1, 10-*Magnaporthe oryzae* 70-15, 11- *Verticillium dahlia* VdLs.17, 12-*Pyrenophora tritici repentis* Pt-1C-BFP, 13-*Colletotrichum gloeosporioides* Nara gc5, 14-*Cladophialophora carrionii* CBS160.54, 15-*Cladophialophora yegresii* CBS114405, 16- *Cyphellophora europaea* CBS101466, 17-*Capronia coronate* CBS617.96, 18-*Exophiala dermatitidis* NIH/UT8656, 19-*Talaromyces marneffei* ATCC 18224, 20- *Talaromyces stipitatus* ATCC 10500, 21- *Arthroderma otae* CBS 113480, 22- *Trichophyton rubrum* CBS 118892, 23-*Microsporum gypseum* CBS 118893, 24-*Coccidioides posadasii* C735 delta SOWgp, 25-*Blastomyces dermatitidis* SLH14081, 26-*Histoplasma capsulatum* NAm1, 27- *Paracoccidioides brasiliensis* Pb18, 28-*Paracoccidioides sp. Lutzii* Pb01, 29-*Aspergillus clavatus* NRRL 1, 30- *Aspergillus fumigatus* Af293, 31-*Neosartorya fischeri* NRRL 181, 32- *Aspergillus niger* CBS513.88, 33- *Aspergillus flavus* NRRL3357, 34-*Aspergillus oryzae* RIB40

activation. Thus, Rad9 binds to proteins mediating the process as Bcl-2/Bcl-x, which consequently activates Bax, initiating the cascade of apoptosis. This connection is possible because Rad9 protein changes its conformation, exposing its BH3 domain, which interacts with the groove of the Bcl-2/Bcl-xL protein which, in turn, is able to activate the Bax/Bak protein. The proteins belonging to the Bcl-2 family are considered anti-apoptotic and the interaction of Rad9 with these proteins presumably regulates negatively this pathway, antagonizing them [35].

The organisms that make up the outgroup, *Homo sapiens*, *Arabidopsis thaliana*, and *Drosophila melanogaster*, display the Rad9 domain (Table 1). This domain is responsive to DNA repairs due to damage, besides having a homologous BH3 domain.

Analysis of the splits graph (Figure 10) does not suggest recombinatory events and phylogenetic trees were obtained. These were confirmed by examination of the RDP, but no significant events were obtained. The tendency to experience positive selection could explain the differences between organisms. From the value obtained in the Tajima statistical test, 3.698058, a positive selection of many codons was expected. However, the analysis by the selecton server (Figure S5) did not confirm this result. According to the results obtained, the protein is under negative selection, which indicates its importance at the structure and/or function of the protein and does not allow changes. This conservative trend seems to be ubiquitous among the proteins of the apoptosis pathway, making them interesting molecular targets for fungi control.

Conclusions

The evolutionary analysis of the pro-apoptotic proteins showed their conservation among the various organisms. Also, our data explain the evolutionary distance from *Aspergillus fumigatus* to other homologous components of the phylogenetic trees. This analysis, together with the study of the domains, and the presence of possible recombinations, allow a better understanding of the importance of the different proteins studied. Thus, we suggest that all the five proteins studied are involved in the apoptotic process of *Aspergillus fumigatus* and are related to each other.

The results of the analysis of the five proteins suggest the possible presence of the apoptosis pathway in the fungus *Aspergillus fumigatus* (Figure 11), but it is known that this route is very complex and that other proteins might be part of this pathway. Furthermore, the phylogenetic analysis indicated that the apoptotic pathway is conserved in the family Aspergillaceae (Figures 12-15). Therefore, more studies should be conducted to better understand the pathway and its conservation in this family.

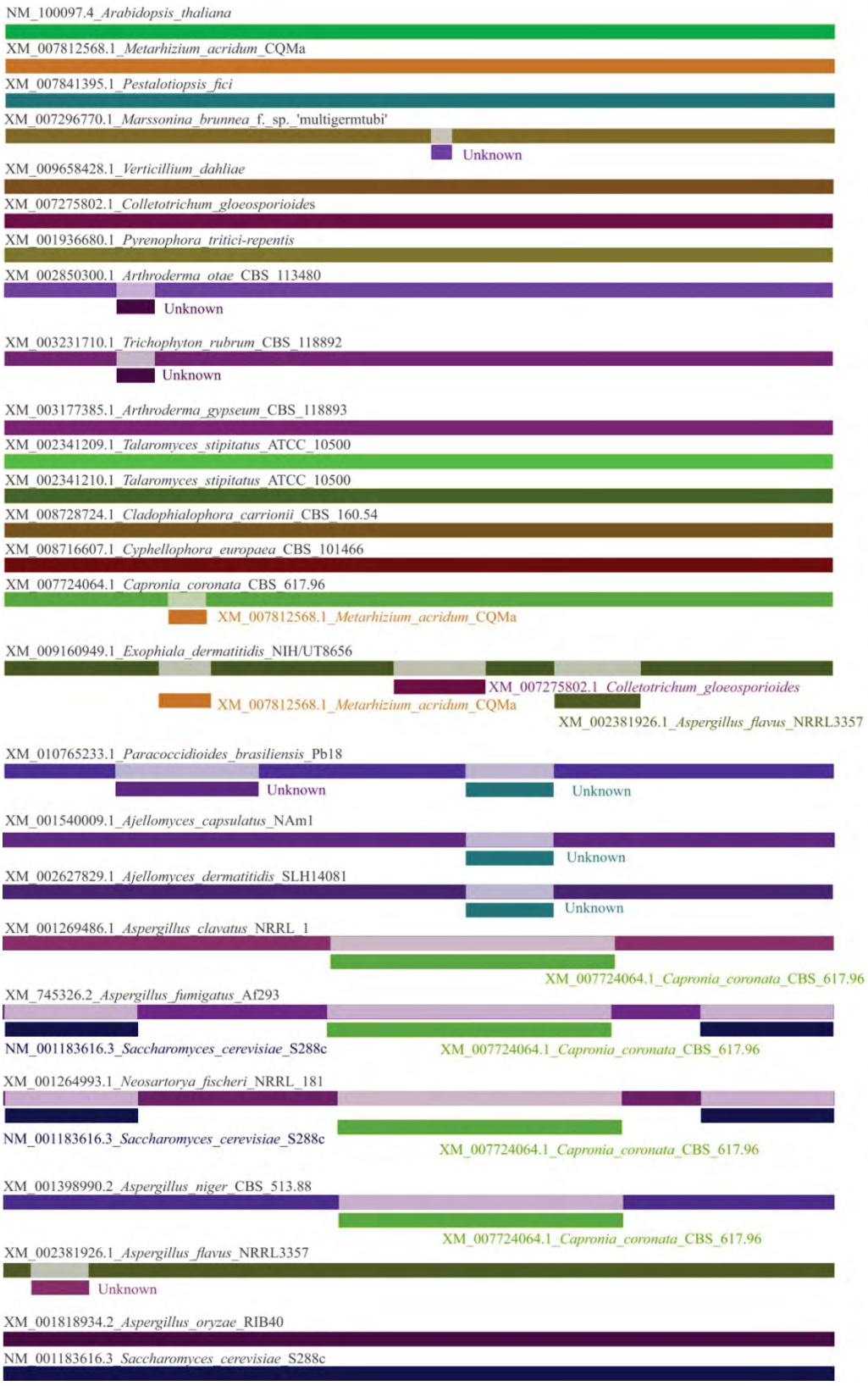

Figure 12: Recombination events involved to Pca1 evolution. Each sequence is represented by a color and the recombination is evidenced by the donor.

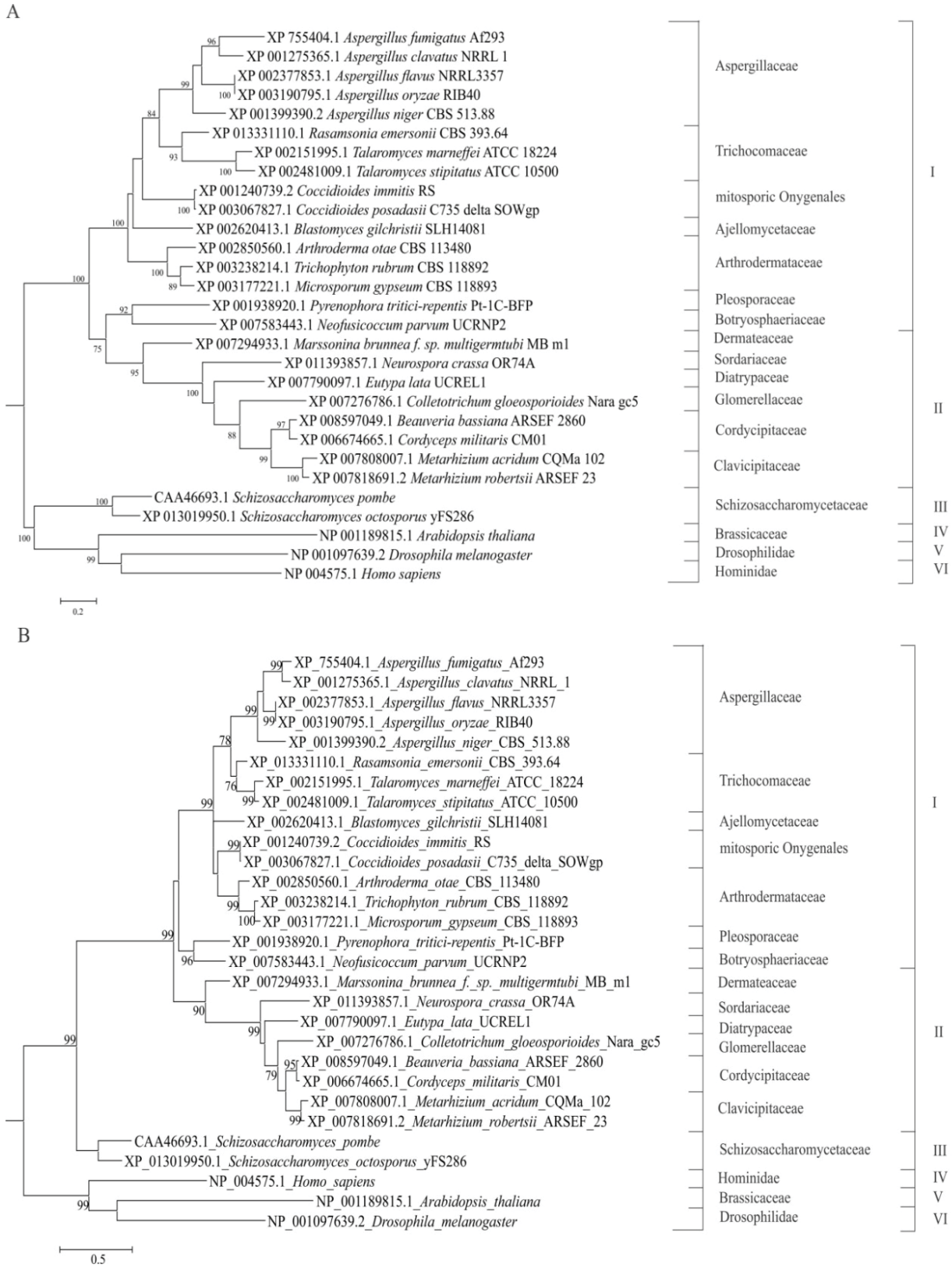

Figure 13: Molecular phylogenetic analysis by Maximum Likelihood (ML) method. The evolutionary history of Rad9 using complete Rad9 protein (A) and G-block cured Rad9 protein (B). All positions containing gaps and missing data were eliminated.

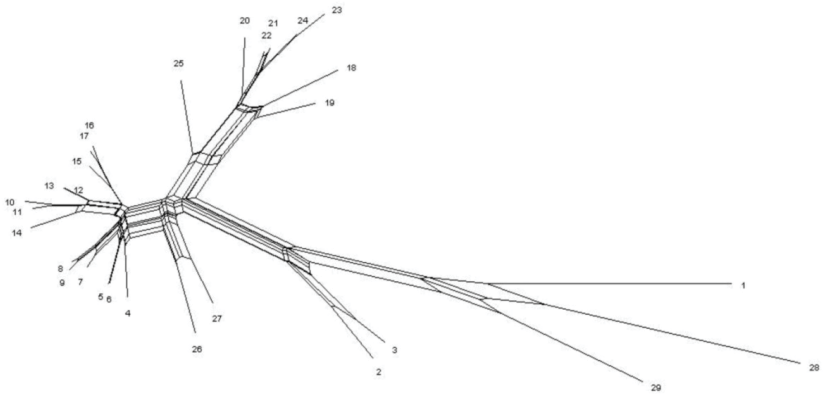

Figure 14: Splits graph of Rad9 protein. The uninformative sites of thrift, gaps and sites listed were excluded. One thousand (1000) pseudo replications were made to sustain the high reliability. We used the model ProteinML correction. The homologous organisms are represented**:** 1-*Arabidopsis thaliana*, 2- *Schizosaccharomyces pombe*, 3-*Schizosaccharomyces octosporus* yFS286, 4-*Blastomyces gilchristii* SLH14081, 5-*Coccidioides immitis* RS, 6-*Coccidioides posadasii* C735 delta SOWgp, 7-*Arthroderma otae* CBS 113480, 8- *Trichophyton rubrum* CBS118892, 9-*Microsporum gypseum* CBS118893, 10-*Aspergillus fumigatus* Af293, 11-*Aspergillus clavatus* NRRL 1, 12-*Aspergillus flavus* NRRL3357, 13-*Aspergillus oryzae* RIB40, 14-*Aspergillus niger* CBS513.88, 15-*Rasamsonia emersonii* CBS393.64, 16-*Talaromyces marneffei* ATCC18224, 17- *Talaromyces stipitatus* ATCC10500, 18-*Neurospora crassa* OR74A, 19-*Eutypa lata* UCREL1, 20- *Colletotrichum gloeosporioides* Nara gc5, 21-*Beauveria bassiana* ARSEF2860, 22-*Cordyceps militaris* CM01, 23-*Metarhizium acridum* CQMa 102, 24-*Metarhizium robertsii* ARSEF 23, 25-*Marssonina brunnea f. sp. Multigermtubi* MB m1, 26-*Pyrenophora tritici repentis* Pt-1C-BFP, 27-*Neofusicoccum parvum* UCRNP2, 28- *Drosophila melanogaster*, 29-*Homo sapiens*.

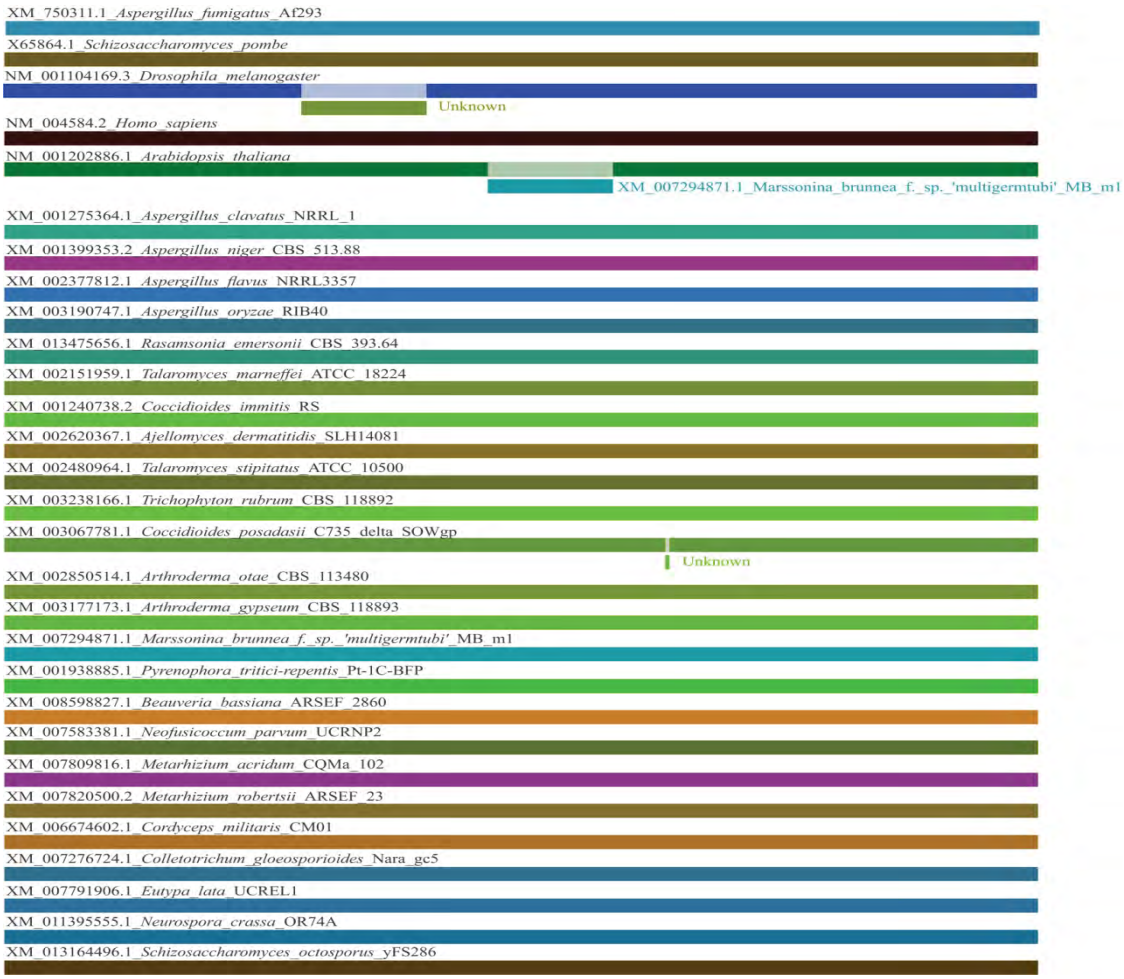

Figure 15: Recombinational events involved to Rad9 evolution. Each sequence is represented by a color and the recombination is evidenced by the donor.

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