

## Supplementary Material (online)

### Mutations in the Second Alternative Oxidase Gene: A New Approach to Group *Aspergillus niger* Strains

Michel Flippi <sup>1,†</sup>, Alexandra Márton <sup>1,2,†</sup>, Vivien Bíró <sup>1,2</sup>, Norbert Ág <sup>1</sup>,

Erzsébet Sándor <sup>3</sup>, Erzsébet Fekete <sup>1,\*</sup> and Levente Karaffa <sup>1</sup>

1 Department of Biochemical Engineering, Faculty of Science and Technology,  
University of Debrecen, H-4032 Debrecen, Hungary

2 Juhász-Nagy Pál Doctoral School of Biology and Environmental Sciences, University  
of Debrecen, H-4032 Debrecen, Hungary

3 Institute of Food Science, Faculty of Agricultural and Food Science and  
Environmental Management, University of Debrecen, H-4032 Debrecen, Hungary

\* Correspondence: kicsiszoka@yahoo.com

†These authors contributed equally to this work.

**Figure S1:** Schematic overview of branched respiratory electron transport chain in the mitochondrial inner membrane in filamentous fungi to illustrate the metabolic function of alternative oxidase (Aox) and type-II NADH dehydrogenase (And).

**Figure S2:** The intron-exon structures of the two paralogous genes for alternative oxidase and for alternative NADH dehydrogenase (oxidizing cytosolic NADH) extant in *Aspergillus niger* strain ATCC 1015.

**Figure S3:** The taxonomically aberrant position of the Onygenales AoxA clade is not a consequence of joint analysis of (orthologous) AoxA proteins with AoxB paralogs.

**Figure S4:** Alignment of opposite LTR's of the non-autonomous and autonomous copies of ANiTA2 found amongst sixteen *A. niger sensu stricto* isolates with *aoxB* disrupted by retrotransposon insertion.

**Figure S5:** Ty1/copia retrotransposon ANiTA2, autonomous copy 5129 nt.

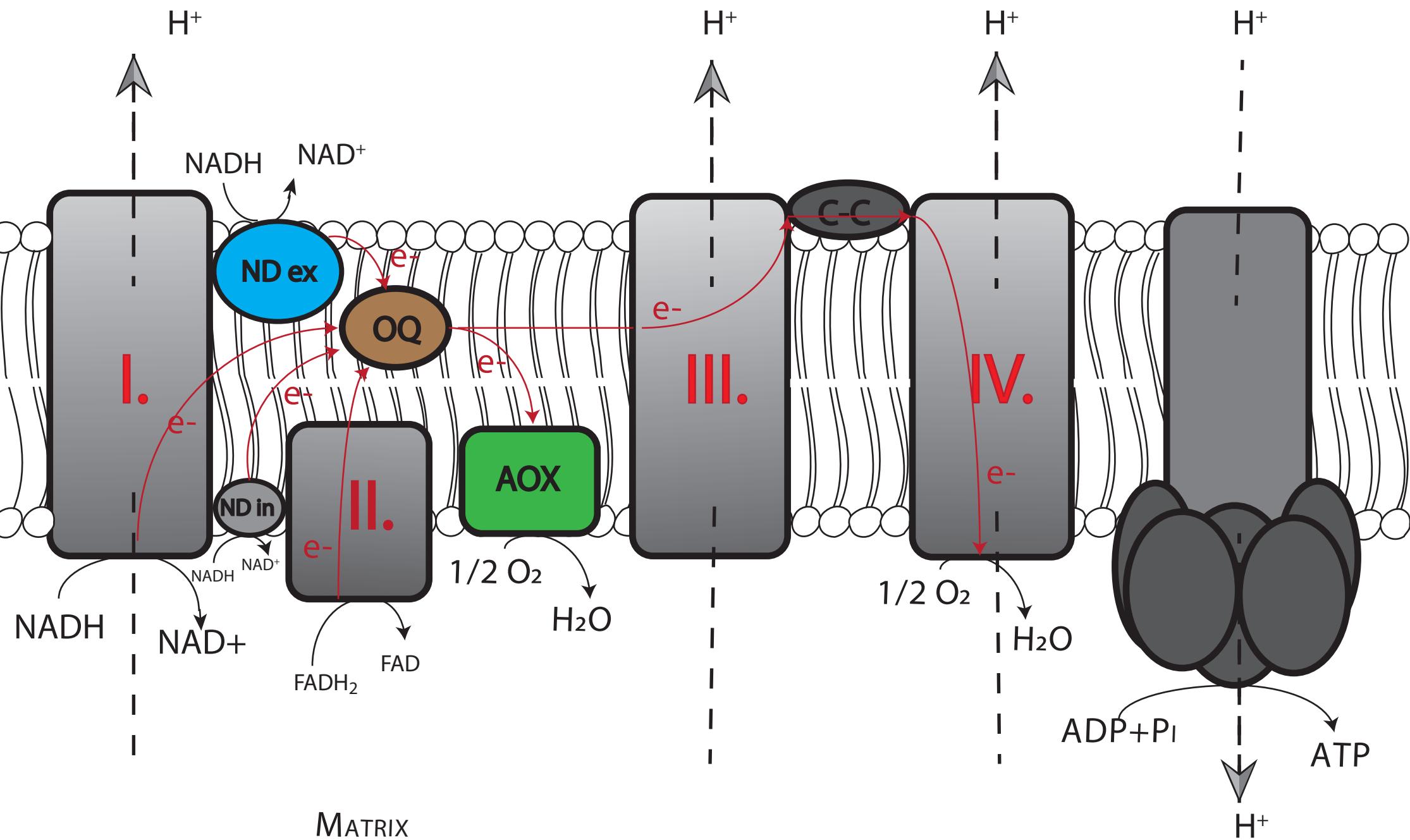
**Figure S6:** DNA similarity between the coding region of *A. awamori aoxB* and the *aoxB* pseudo gene in *Aspergillus niger (sensu lato)* strain An76.

**Table S1:** The intron-exon structure of *aoxB* and divergently orientated *andB* genes.

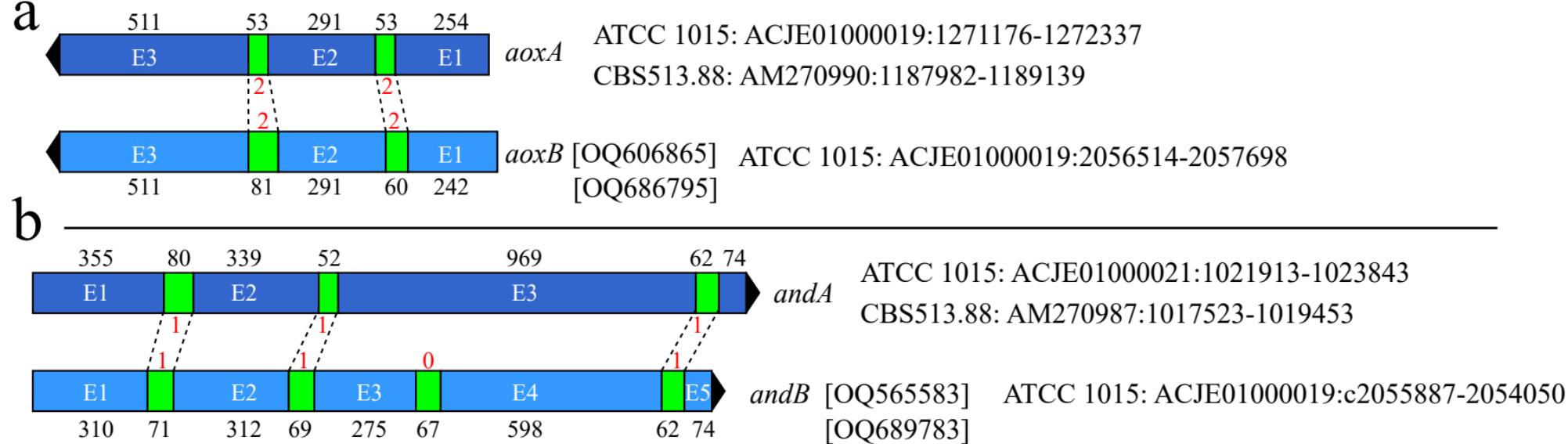
**Table S2:** NCBI-lodged RNA sequence reads that cover exon-exon fusions in *aoxB* and *andB* in *A. calidoustus*, *A. implicatus* and *A. niger* strains (with full-length *aoxB* and *andB* genes).

**Table S3:** Oligonucleotide primers used for PCR amplification of the *aoxB* and *andB* genes and cDNA prior to sequence verification of mutant alleles.

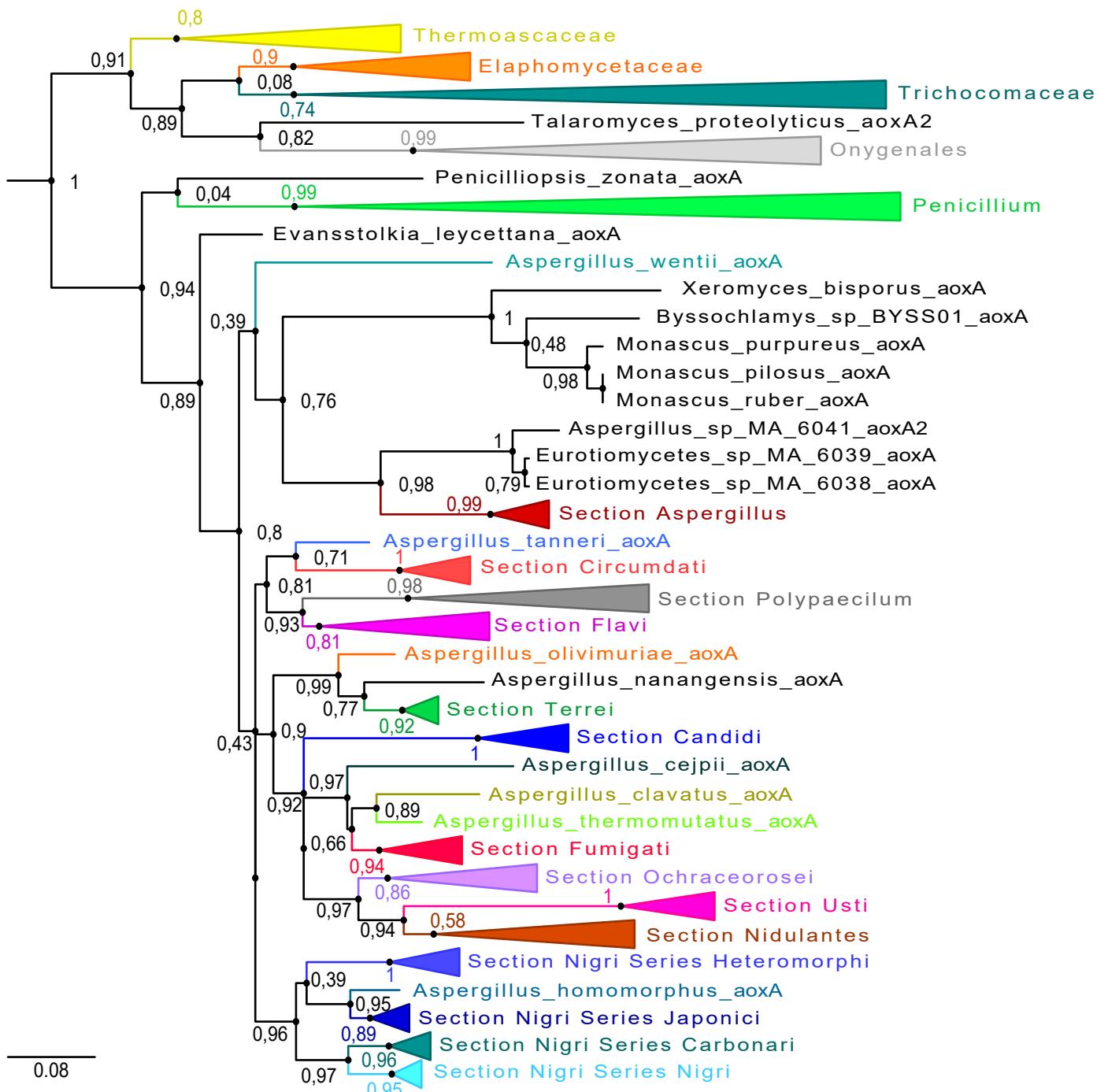
## INTERMEMBRANE SPACE



**Figure S1.** Schematic overview of branched respiratory electron transport chain in the mitochondrial inner membrane in filamentous fungi to illustrate the metabolic function of alternative oxidase (Aox) and type-II NADH dehydrogenase (And). The alternative electron fluxes are indicated in red. Associated translocation of protons over the inner membrane are shown. In the canonical chain, Type-I NADH:ubiquinone oxidoreductase (complex I), Ubiquinol:cytochrome C oxidoreductase (complex III) and Cytochrome C oxidase (complex IV), respectively, couple electron transport down the chain to proton translocation and the buildup of proton motive force that can be used to generate ATP by oxidative phosphorylation (at the right of the scheme). Complex II (succinate dehydrogenase) re-oxidizes mitochondrial FADH<sub>2</sub> formed by the prior conversion of succinate to fumarate in the tricarboxylic acid cycle without pumping protons. Alternative oxidase (Aox; green) accepts electrons directly from the intermediate carrier ubiquinol (brown) and transfers them straight to molecular oxygen, bypassing the electrogenic, proton-translocating Complexes III and IV. NADH/NAD<sup>+</sup> cannot pass the mitochondrial inner membrane. Alternative NADH dehydrogenases (And) or Type-II NADH:ubiquinone oxidoreductases oxidize either cytosolic (blue) or mitochondrial NADH (grey) and transfer the high-energy electrons to the ubiquinone intermediate (brown) without concomitantly pumping protons. Type-II alternative NADH dehydrogenases thus bypass the proton-translocating (Type-I) Complex I



**Figure S2.** The intron-exon structures of the two paralogous genes for alternative oxidase and for alternative NADH dehydrogenase (oxidizing cytosolic NADH) extant in *Aspergillus niger* strain ATCC 1015. **(a)** Alternative oxidase genes, *aoxA* and *aoxB*. **(b)** Alternative NADH dehydrogenase genes, *andA* and *andB*. The exact location of the four genes in *A. niger* strain ATCC 1015 (sequence contig and coordinates) is mentioned to the right of the gene models. Relevant GenBank accession numbers are included. For the two ubiquitous “A” genes, the exact localisation in *A. niger* strain CBS 513.88 is also given. The exons of the coding regions (i.e., between ATG and STOP) are colored darker blue for the ubiquitous paralogous genes “A” and lighter blue for the rare paralogous genes “B”. Introns are indicated by the green color and vertically connected with the dashed lines to emphasize intron position conservation. The arrowhead gives the orientation of the genes. The intron phase is indicated by the red digit (0; 1; 2). The phase-zero third intron is lost from the ubiquitous gene for the alternative NADH dehydrogenase, *andA*.



**Supplementary Figure S3.** The taxonomically aberrant position of the Onygenales AoxA clade in maximum likelihood estimations of interrelations of Aox paralogs is not a consequence of joint analysis of (orthologous) AoxA proteins with AoxB paralogs. Essentially the same clade clustering was evident from control phylogenies of exclusively the AoxA input (i.e., with the AoxB paralogs omitted). The Figure shows such a “AoxA-only” control tree constructed using exactly the same methodology for multiple sequence alignment (MAFFT), its trimming (BMGE) and the subsequent maximum likelihood inference (PhyML) (cf. legend to Figure 4b). The rooting outgroup of Lecanoromycetes AoxA was eliminated from view by subtree selection in FigTree. The clades specific to Thermoascaceae, Elaphomycetaceae, Trichocomaceae, Onygenales, the *Penicillium* genus, as well as the various *Aspergillus* sections were collapsed for a more convenient overview. However, section Nigri species were collapsed at the series level. Note the considerable evolutionary distance between the section Nigri series Nigri AoxA clade (at the bottom of the tree), the section Usti (includes *A. calidoustus*) and the estimated origin of paralogous AoxB as a sister clade of the Onygenales AoxA, near the top of the tree.

**Supplementary Figure S4.** Alignment of opposite LTR's of the non-autonomous and autonomous copies of ANITA2 found amongst sixteen *A. niger* sensu stricto isolates with aoxB disrupted by retrotransposon insertion (aoxB allele III)

[118/162] 72.84 % ID [copy disrupting aoxB]  
  
ATTTAATGTTGAGGCAGTAACCCTGTAGGCTCAGTCAGTGGCTCGCCTCAGGCCACCAAGCGCTTCAGCGCTGGTTGCGCTGCGTGGTTCTAGATAGTAATATAACTTTACTATATTCTCACCTTCGTTCTCCGCCTGAGTATTGAATAGA-----  
-----TGTTGAGATAGTAATTCTATAACTTAATTATGCTCAGACGCCACCAAGCACCTTGCAGTGCCTAGATAGTAATACAACACACTTTACTGCATTCTCGCTTCGTTCTCCGCCTGAGTATTAAACA[GAGAATG]  
  
[131/162] 80.86 % ID  
  
AGCTTTGTGAGGCAGTAACCCTGTAGGCTCAGTCACGTGGCTGTGCTCAGGCCACCAAGCGCTTCAGCGCTGGTTGCGCTGCGTGGTTCTAGATAGCAATACAACACTTTACTGTATTCTTGCTTTGTTCTGCTGGTAGCGTTGAATAGATT-----  
-----TGTTGAGATAGTAACCTATAGACTCATGTAGCTGCCTCAGGCTACCAAGCGCTTCAGCGCTGGTTGCACTGCGCTTCAGATAGCAATACAACACACTTTACTGCATTCTCGCTTCGTTCTCCGCCAGCGTTGAAC[AGCTTTGT]  
  
[141/164] 85.98 % ID  
  
CATTAGATGTTGAGGCAGTAACCCTGTAGGCTTGGTCACATGGCTCGCCTCAGGCCACCAAGCGCTTCAGCGCTGGTTGCGCTGCGTGGTTCTAGATAATAACACATACTTTACTATATTCTTACTTTCTATTAAATAATTTAAATAGA-----  
-----ATGTTAAAGATAATAATTCTATAAAACTCAGTTATAGGCTATGCTCAGGCCCTACTAACGCGCTTCAGCGCTGGTTGCGCTGCGTGGTTCTAGATAGTAATACAACATACTTTACTGCATTCTCGCTTCGTTCTCCGCCAGCGTTGAAC[CATTAGATG]  
  
[125/163] 76.69 % ID  
  
AGTTTATTGAGGAAGTAACCCTGTAGGCTCGGTACGTGGCCGCGCTCAGGCCACCAAGCACCTTCAGCGCTGGTTGCACTGCGTGGTTCTAGATAGTAATATAACACATACTTCACTGCATTCTTGCACTGTTCTTCTATTAGTTGAATAGAT-----  
-----TGTTAAAACAGTAATCTATAAAATTAAACTATGCTCAGGCCACCAAGTACCTTTACTGGTTGCTGTATATTCTAGATAGCAATACAACACTTCACTGCATTCTTGCGTCTTCCACCTGAGTCGAAC[AGTTTATT]  
  
[116/161] 72.05 % ID  
  
-ATCAGATGTTGAGGCAGTAATCCTGTAGGCTTGGTCACGTGGCTCGCCTCAGGCCACCAAGCGTTTCAGCGCTGGTTGCGCTGCGTGGTTCTAGATAGTAATATAACTTACATTCTCGCTTCGTTCTCCGCCTGAGTGTAAATAGA-----  
-----ATATTAAAGATAGTAATTAGTTAGTTAGTGTGGCTGTGCTCAGGCCCTACTAACGCGCTTCAGCGCTGGTTGCGCTGCGTGGTTCTAGATAGTAATATAACTTACTGCATTCTCGCTTCGTTCTCCGCCTGAGCGTTGAAC[ATCGAT]-  
  
[134/163] 82.21 % ID  
  
-AAATCTGTTGAGGCAGTAACCCTGTAGGCTCGGTACGTGGCCGCGCTCAGGCCACCAAGCGCTTCAGCGCTGGTTGCGCTGCGTGGTTCTAGATAGCAATACAACACATACTTTGCTGCATTCTTGCGTTATTCTCCCTTTGTAATTAAATAAA-----  
-----TGTTAAGACAGTAATCCTATAAGTTAACATGCCACGCTCAGGCCACCAAGCGCTTCAGCGCTGGTTGCGCTGCGTGGTTCTAGATAGCAATACAACATACTTTGCTGCATTCTTGCGTTCTCCCTGTAGTCAAACA[AATCT]-  
  
[142/160] 88.75 %  
  
AGAAACGTTGAGGCAGTAACCCTGTAGGCTCGGTACGTGGCCGCGCTCAGGCCACCAAGCGCTTCAGCGCTGGTTGCGCTGCGTGGTTCTAGATAGCAATACAACACACTTTGCTGCATTCTTGCGTTCTCCCTGTAGTCGAACAG-----  
-----TGTTGAGGCAGTAACCCTGTAGGCTCGGTACGTGGCCGCGCTCAGGCCACCAAGCGCTTCAGCGCTGGTTGCGCTGCGTGGTTCTAGATAGCAATACAACACACTTTGCTGCATTCTTGCGTTCTCCCTGTAGTCGAAC[AGAAAC]-  
  
[159/159] 100 % ID [autonomous copy ANiTA2: Ty1/Copia family]

Recombined LTRs (solo LTR with TSDs)  
[autonomous copy recombined out: solo LTR 100 % identical

The target site duplication (TSD) directly upstream and downstream of the transposon LTR's is highlighted by the green background.

**Supplementary Figure S5.** Tyl/copia retrotransposon ANiTA2, autonomous copy 5129 nt

AGACTGAGGCTGAGAGCGAGTGCACTGGCTCGCTGCAACCGTGGCTTCGAGCAGCTAACGATGCACATCCGGTGGCAAAAGATTGGCAAAGAAACAGTTGAGGCTAACCCTGTAGGCTCGGTACCGTGGCCGCGCTCAGGCCGCCACCAAGCGCTTCTCGCGCTGGTTGCGCTGCCGTTCCCTAGATAGCAATACAACACACTTTGCTGCATTCTTGCCTTGTAGTTGCGAAGCAGttatgagccggcctctgcgt  
gaactcgattcactcgactcgactcgatccggggccaaacggacggggccatcgcccaaaacggatcggttgcacggacgcgttgttgcgttgttgcataatccaataagaacaccctttgcgcggatggatcac  
catcgctcgatcccgatcgacttgcataatggcageATGCAATTGCGAAGCGCCGCGTTCTGGCTTAAGAACCTTCTCGCTGAAGGGGAGGCTACACCGCAGGGGATAGGCTACCCGCCAG  
ATGCCCTGATGGCTTCTGGCTACCTCTCTGCTCACCGCGAAACACTTCTCAAATGGGACTTCCAGGTGAAGCAACCACTTAAGAACGTCGGCTTGGAAAGACCTGATCAAACACTGGACCTGCCCAGC  
ACGAAAAGTTCAAGCATGGCACAGTTACTCCCGTGTCTACAATCTGGTTGACGGGCCAGCTATGGCAAGGTGATGGACCACTTCCAGGGTCCGCTGACAAAAGGACTATGTCGACGATCATATAAGCAATACGAAG  
AATCGTGATGGCCACGAAATTGCTCTGCCCCAGGGCTTACACCTGTTAAACCGAAGCGCTGGACTATGCAACGGGGTGTATTACATACAGGATTCTGGAATAACTATGCTCTAGCAAGGAGTTGAGGTGTGG  
ATACCCCCCTTACAGCCTCGCTACTGCTCTCCGGAGAGATCCAACCGATATGCCGCTGGGGCACCACATGGAATGCAAGATGCCGAGAATGCAAGACTCTGTACAGATAATGACTTCTTACTTTGAGAAACG  
CTATTGAACAGATAACAGAATAACGATATGGATTGCCGCACTACAAAAGCGGAAACCGGATCTGTCATACAAATCAGATCTGCAAGGGGATGACTGGAAGAAGGCAACCTACCGGAAAGAGGTGTTCAATGAAGA  
CCACGCGCAAAAGATGCGAAATAAAAGCGCAGTATGTTGACGGAGCTTGCCTGCGTTACTGCGAGATGCAAGGAAAGTCATGGTCTCCGCAAGTGTGGTATATTGAAACCAACCTTGTCCACAGGCTGGGTGCTTATAATGGAT  
ATTGGTTCTCCGACCCAAAGGTGATCGTGGTAAGCGGAAGCAGGAAACAAAAGATGACTTAAAGCGTTGAGGCGACCTGCAAAAGCAATCGTCAAGGATGTCGACTGCCAACAGCTGCAAGCGGAGTCGGATG  
ACGAAAATTGCAATTAAATTCTGTTGACTCGCCGCTGGGAACACACTGAGGGTCTCTGCGCTGAGTACGATACTGGCAGTTCGCACTGACCTGACCGGCTCGTCAAAACAACCTACAGGACCTGGATG  
CTGCAACTTCTCAAGTATGGCACCTCTGATGGTGGCTGGTGTGCAAAATCAGCAGGTTACCACTGACTGTTCTGTTGCTTAAGAACCCCTTGTGACTCAAGATAAAAGCTTACTACAGCCGGACTGCCCTACGGCTC  
CTTTCAGCGGACCGCCTGCGCAGGGATTTGGTATACGCCAACACGAAAGACCTCACCGAACAGGATGACAAAGTGGTGGCCACCTGCAAGCATGCAAAAAGCTGTTCTGCAACCGGAAGTGCAGGGC  
TGGCTCTGGCTGAGTGGACCCCTACTCTGCACCGGCTACGGCATGCAAGGTTCCGCAAGGTCAGGTACCTCCGCTGCGAAAAATCAACATATTGAACTGCAACACCGTGAACGACTCCACTGCGAAGCGTGTCCCTAGGAA  
AGCGAAGCGGTTGGTATCCAGAGATCGATCCCGAACGCGCTGACCCCTACCCAGCTGTGGCAGCCTGACGTACAGCAAGTGACCCCAATCGGCACGGAGGCTCAATACTTCTGGTCAATGCAAAACGCTACTCGTTG  
GTCCGTACGGCATGCTGAAGCAGAAGAACCGCCTACGGAGCTGATCCGAATGAATAACGAACACAAGAATATGGTGGCCGCTACGGTCCCTCGACGGGGCCCTGTAATTAAATTCAAGAGTGG  
CCAACCCACGAAGATAAGGAAATTGTTGATATGCCCTCTAGAACACCTGAAAGGCAACGCCCTGCTGAGCCTACGGCGCTGAGCAGTCAAAACACCGCTTCTAGATGCTGACTCCGACTACCGGTGACCTCTG  
GCCGTTGCGGTACGCTGGCGCAGATACTGAAATCGGTTGGTCTCTAGCGGAAGCAGCAGTCAAAACACCGCTTCTAGATGCTGACTCCGACTACAGTCAACCTCCGAAATCCGATGCCACCCGACTTCTACGTTG  
GGATGCCGCTGCTACACACATACCGGTAGAGGACCGCCTGACCTCCGAAAGATGGGGCCCGCCGCGTACTGGTTACTTGTGGCTGACGGGGCACGCCACGTGTTCTGCTGGTATCCTGCTGCAATAAGG  
TGAAGAAATCGGTGACGTTACGTTCTATGAGGATGCGACGTATAAGGATGATCACGAAGATCTGGTAGAACCGCTGACGGCCACTCTCAAGGGTCCGATCATACGGAGTCAGGCACCTGATCAATTGATAACACAAT  
TCTCGTGCATAAAAGAATACAGAAATTGATAACGCTAACGCCCTCCCTACACAGAATGACACCGCTGACGAACCCGTGCGCGGAAGAATACAGACTTATGAGTTACTGAAACCTCCGACCTACCGGACCAATCCGCTCG  
ATCGAGAATCCGCCGATTACGAGGCTCGTCTACAAATACGCACTACGAGACTTCCGCTTGTGATTGGAGCCCCCTACTAAGGCCGTTGCTACCGCTCCAAAGCAGATCTAACAAAGGAAAGAGTCC  
AGTGGCTAGGAAATGAAACAGTGGCTGACTGGAGCAGGAATCCAACCTACCTCCCTGCTGCACTGAAACACAGAAGTACTGATACCGAACACCTATACGAGGGCATTTCGTCACCGCTCGCTCGTACTGGAGGCGC  
CATGACCCACACAGATGATAAGTCAAATCAATGATGATCGTTGACGGCCCTGCTGGCGCTCGTGTGATCCGGCAAGTGGTGTGTTGACCTGAAAGTCAACCCGAAGGTGTTATTACAAATTCTGCTCGT  
TGGTTGTTGTTGGAACACGCCGAGTGGCTCCGGGATCGATTACGAGTCTTCGCCCCCTGCGGAGATCGCCTGACCGGACTACAGTCAACCTGCTGAGGGTGTGTTCTGCTGCTGCTGCTG  
CATACCTCCACCGCTCAGATTGAAAGTAAACCGTGTATATGAAACCACTACAGGTTATCAGGAAGGGCACCTGGGGCGCTGCTGCTGTTGAAATGGCTCTACGGACTTCCGCAAGCCGACCTCTGGAAACCCGACCTT  
AGACGAAAAGTTACAGGAGATTGGTTCAACACCTGCCCAGAACCCCTGCGTGTACAGAATGGGATATATGGCTGATAATCTATGTTGATTCGCTATCGCAGGGCAGAAGAGGAGATCTAACCTCGGTAAGGGT  
CACCTTAACGAGCGTTCGGCTCAAGGAAATTAGCGAACCGAAGGGTCTGCTAGGGTCAATGTTACGATACGAAACCGGACCTTCACATCCAAACCCAGCTATGTTGAGGAGTGGCTAGCTGCCGTAACCTAC  
AAGCTGCTCTGGCTGCTATTCAATGACCCCTACCTACAAGAAGGGGATCGTACAACAATGACGACTGATAATGTTGATATTAACGAATACAGACGCTCGTGGTAGGCTGATTGGTTGAGCACGAAATCACGCCCTGA  
TCTCGGCCACGCTACCTTCCGCTGACAGAGAAGGGATGACGATCCCACTACCGCTGACGTAAAGCACTGACGAGACCGTGTACAGATATCCGTGACACCGTTCTATGGGATCCTCTGGCCACCGATACCCAAAGGAATC  
GAGGTCTACGTCAGGCCGCGCATGCGGATCACGAAGATGGCAAGTCCACTGAGGGTTACGTTATCTCTACACCGGTGGCCCTATACCTGCTGCAACAGCAAAAGCGTTGCGCCCTCTACCGTTGAGAATACT  
GCCGATTGATGCGAGCGCTCAAGGACGCTTATATGTCAGGAAAGCTGCTGGCCCTTGTGACCTCAAGGATATCGGATGACGGCAAGGAAACCCCTGTTAGCGGACGCCGCTAATTCGCTAAAGGATATTGAAACCATGGCGCTACAC  
CAAGTCGACTAGATGGCTCGATAATCGCTACCTATTGCGGCCGATCTAATCAAAACAGCAACGCTATATCAACACGTTGAGGTGTTCCAAACCCCTGCTGACGGCCCTACTAAACCCGCTGGATCGGGAAGCGTCC  
TTCAAGCGCCCTGCTAGGAATGACCCCGATCGCAGGCCACCGATCTGCTGACCTGGAAACTAGacttaatatgtgc当地atggaggaaagtacggcttc当地accccttgc当地cacgttccgc当地taggggg  
gTGTGAGGCAAGTAACCCCTGTAGGCTCGGTCACTGCGCCGCGCTCAGGCCGCCACCAAGCGCCCTCTTCCGCTGGTTGCGCTGCGCTTCTAGATAGCAATAACACACACTTTGCTGCTATTCTTGCCTTCTC  
CTGTAGTTGCGAACAAGAAACAAAGTCGACAGGAAAGTGAGTGGCGGTGATGAACGGAGACTTGATTGATTGAACTGCCAGGGACGGGGTCCCATGACATCACCGGCCACCGTCGGGA

LTR = 159 nt (purple: 5' & 3' = 100 % identical); TSD = 6 nt 5'-AGAAC (green).

ORF = 4512 nt (yellow); Integrase-RT-RNaseH polyprotein (Tyl/copia order) = 1503 AA

**Supplementary Figure S6.** DNA similarity between the coding regions of *A. awamori* aoxB and the aoxB pseudo gene in *Aspergillus niger* (*sensu lato*) strain An76

BDHI_Aawamori	ATGGTACGCCAAATGCCATCCTTTCA	GCAGCCGATAACCGAAAGAGGTCAATGTACCTT	60
BCMY_An76	ATGATACGCCGAATGCCGTCCTTCA	GTCAGTCCGATAGGGCGAAAGTAGGTCAATACATCCT	60
BDHI_Aawamori	ACAAACATGCATCGCATTCATTGCA	AAACAGACCAGAAAAGCCCCTTAATCACACAG	120
BCMY_An76	CAAACATGCATCGCATTCATTGCGCAATAA	ACCAGGAGACCACGGAAATTAAACACAA	120
BDHI_Aawamori	CTCATACTTCTTGCTGGACAAGCGGGCTT	GGCACCTGAACTCCTTATCATTCAAGTTG	180
BCMY_An76	CGATACTTCTTGCTGGACAAGCGGGCTT	GCTACCTGAACTCCTTATCATTCAAGCTG	180
BDHI_Aawamori	CAATGCCACCAACAAAAT	TGACCCGAATCAAGGAGAGAGCTATTGCTGGAAACACCCA	240
BCMY_An76	CAATGCCACCAACAAAATCA	ACCCGAATCAAGAAGAGGCCATTGCTGGAAACACCCA	240
BDHI_Aawamori	ATgtcagccatatactccctttactttt	tgtgtctgtcttccttaactac---	295
BCMY_An76	ATatgagttcacctatcccttttttttgg	gtgtctgtcttcctctgtcttccttcctgccttcctg	300
BDHI_Aawamori	-----ggaa	aagCTATACAGAAAAGGAGATTGAAATATCACTGTAGCTCATCGCGA	348
BCMY_An76	acaattaaaaaaaac	CTATACAGAGAAAAGAGATTGCTGATATCACTGTGGCTCATGGCAA	360
BDHI_Aawamori	AGCCAATGCCCTTCTGACAAGATCGCCTT	GGGCTTGGTACGTTTGCTTCGATGGTGCAC	408
BCMY_An76	TGCTAACGTTCTTCTGATAAGGTCGCC	TTGGCTTGGTACGTTGTCTTCGATGGTACAC	420
BDHI_Aawamori	TGATTTCTGACTGGTATCGAAATCCC	GAAAGGAAAGAAATCGCAGACAAGTTGA	468
BCMY_An76	TGATTTCTGACTGGTATCGAAATCC	GAAAGGAAAGAAAGTCGTAGACAAGTTGA	479
BDHI_Aawamori	TATGACTGAGAGAAATGG	CTTATTCTGTTCAATTCTCGAACATCGAGACAGTTGCTGGTGC	528
BCMY_An76	GATGACTGAGACAAAGCC	ACTTATTAGTTTCACCAATTCTCGAACATCGAGACAGTTGCTGGTGC	538
BDHI_Aawamori	TGGTATGGTGGCGGCATGCTCC	GACATCTGAAGAGTATCAGACGGATGAAGAGGGATCA	588
BCMY_An76	GGGTATGGTGGCGGCATGCTCC	GACATCTGAAGAGTATCAGACAGATGAAGAAGGGATCC	598
BDHI_Aawamori	TGGATGgtcagtacaaggaaaaaa	-t-aatttatctccaagtacttagcagtagctaca	646
BCMY_An76	TGGATGgtcagtgcaggaaaaaa	ataaaatatcttcaaaatgttaaaagtagctaca	658
BDHI_Aawamori	caagaagctgcagactgac	acaacccgcagGATCGAGTCCTTGATTGACGAAGCCTACAAC	706
BCMY_An76	caagaagctgcgactgac	acaactgcagGATTGAGTCCTTGATTGAAAGCCTACAAC	718
BDHI_Aawamori	GAGCGCATGCACCTTGTACATT	CCTCGAACACTGGCAGATCCAGGTATCTTCATGCGCTTA	766
BCMY_An76	GAGCGCATGCACCTTGTACATT	CCTCGAACACTGGCAGATCCAGGTATATTGCGCTTC	778
BDHI_Aawamori	GTGTCTTGGCGCGCAGGGCG	TCTCTTCATCAATGCCCTCTCTGCTCTACCTTGTTC	826
BCMY_An67	ATGGTCTTGGCGCGCAGGCC	TCTTAAATGCCCTCTTGTCTCTATCTCGTTCTA	838
BDHI_Aawamori	CCCAAGACATGTCACCGGTT	TGTTGGATACCTAGAGGAAGAAGCTGTCATTACCTATACA	886
BCMY_An76	CCCAAAACATGTCACCGGTT	CGTGGATACCTACAGGAAGAAGCTGTCATTACCTTACA	898
BDHI_Aawamori	OACGCAATCCGTCAACTT	CAAGCTGGAAACTGCGCAGCATGGGACAATTATCAGCACCG	946
BCMY_An76	OCTGCAATCCGTCAACTC	CAAGCTGGAAAGCTGCGACGATCACCAGCACCG	958
BDHI_Aawamori	GAGATTGCAATCAAATATTG	GAGAATGCCAGAAGGAAACAGAGAATGGTCTGATTGTTG	1006
BCMY_An76	GAGATTGCACTCAAAGATTG	CAGAATGCCAAAGGGAAACAGAGATGCTTGTGATTGTTG	1018
BDHI_Aawamori	CTTATGTTCGGGCGGAT	GAAGCGAAGCAGTCGCGAGGTAAACCATACTTGGAAACTTA	1066
BCMY_An76	CTCTATGTTCGGATGGAT	GAAGCGAAGCAGTCGCGAGGTAAACCATACTTGGGGAA-	1073
BDHI_Aawamori	GACCAGCATGAAGATCCC	AAATCCTTACTGCGCTTATCACGATGGGATAATGCTGGT	1126
BCMY_An76	---CAGCAAGAGGATCCC	AAATCCTTACTGCGCTTATCACGAAAGGGATAATGTTGGT	1130
BDHI_Aawamori	CCCTCAGCGTCGCCGAT	GCAGGGAAAGCCTATTGGATGGGAGATGAACTAATTAG	1186
BCMY_An76	CCCTCAGCGTCGCCATCG	GCACGGAAAGCCTTTGGGTTAGAGCGAAATGAAAGTAATTAG	1190

Three frameshift mutations are indicated by the hyphen in the red background in the aligned An76 sequence as opposite to the green background in the *A. awamori* sequence. The 5'-donor, lariat branch point- and 3'-acceptor sequences in both introns are given in red lettering. These three sequence elements in the seemingly noncanonical first intron in An76 are in the turquoise background.

**Supplementary Table S1: The intron-exon structure of aoxB and divergently orientated andB genes** from the start to the stop codon. The sequence contig and the coordinates precisely localise (the coding parts of) the exons for each gene in each fungus. The phase and size of the position-conserved introns separating consecutive exons are given. For *Aspergillus implicatus* and *Penicillium swiecickii*, the data refer to the sequence assemblies accessible at the Mycocosm website of the Joint Genome Institute (US Department of Energy). We did not use the results from autoannotation: such predictions prove incorrect in *Aspergillus niger* ATCC 1015, NRRL3 and CBS 513.88 for both aoxB and andB.

		exon1	intron1	exon2	intron2	exon3	intron3	exon4	intron4	exon5
<b>A. niger</b> ATCC1015 andA	NA	ACJE01000021 1021913-1022267 355 bp	phase 1 80 bp	ACJE01000021 1022348-1022686 339 bp	phase 1 52 bp	ACJE01000021 1022739-1023707 969 bp	phase 1 62 bp	ACJE01000021 1023770-1023843 74 bp		
<b>A. niger</b> ATCC1015 aoxA		ACJE01000019 1271176-1271429 254 bp	phase 2 53 bp	ACJE01000019 1271483-1271773 291 bp	phase 2 53 bp	ACJE01000019 1271827-1272337 511 bp				
	ATG-CAT*	exon1	intron1	exon2	intron2	exon3	intron3	exon4	intron4	exon5
<b>A. niger</b> ATCC1015 andB	626bp	ACJE01000019 c2055887-2055578 310 bp	phase 1 71 bp	ACJE01000019 c2055506-2055195 312 bp	phase 1 69 bp	ACJE01000019 c2055125-2054851 275 bp	phase 0 67 bp	ACJE01000019 c2054783-2054186 598 bp	phase 1 62 bp	ACJE01000019 c2054123-2054050 74 bp
<b>A. niger</b> ATCC1015 aoxB		ACJE01000019 2056514-2056755 242 bp	phase 2 60 bp	ACJE01000019 2056816-2057106 291 bp	phase 2 81 bp	ACJE01000019 2057188-2057698 511 bp				
<b>A. awamori</b> IFM 58123 andB	621bp	BDHI01000007 c2238650-2238341 310 bp	phase 1 71 bp	BDHI01000007 c2238269-2237958 312 bp	phase 1 69 bp	BDHI01000007 c2237888-2237614 275 bp	phase 0 67 bp	BDHI01000007 c2237546-2236949 598 bp	phase 0 66 bp	BDHI01000007 c2236882-2236809 74 bp
<b>A. awamori</b> IFM 58123 aoxB		BDHI01000007 2239272-2239513 242 bp	phase 2 61 bp	BDHI01000007 2239575-2239865 291 bp	phase 2 81 bp	BDHI01000007 2239947-2240457 511 bp				
<b>A. welwitschiae</b> CCMB 674 andB	625bp	PUWS01000026 128688-128997 310 bp	phase 1 71 bp	PUWS01000026 129069-129380 312 bp	phase 1 69 bp	PUWS01000026 129456-129724 275 bp	phase 0 67 bp	PUWS01000026 129792-130389 598 bp	phase 1 62 bp	PUWS01000026 130452-130525 74 bp
<b>A. welwitschiae</b> CCMB 674 aoxB		PUWS01000026 c128062-127821 242 bp	phase 2 59 bp	PUWS01000026 c127761-127471 291 bp	phase 2 81 bp	PUWS01000026 c127389-126879 511 bp				
<b>A. calidoustus</b> SF006504 andB	551bp	CDMC01000004 c622233-621924 310 bp	phase 1 49 bp	CDMC01000004 c621874-621536 339 bp	phase 1 67 bp	CDMC01000004 c621468-621194 275 bp	phase 0 73 bp	CDMC01000004 c621120-620526 595 bp	phase 1 73 bp	CDMC01000004 c620452-620379 74 bp
<b>A. calidoustus</b> SF006504 aoxB		CDMC01000004 622785-623020 236 bp	phase 2 52 bp	CDMC01000004 623072-623362 291 bp	phase 2 70 bp	CDMC01000004 623433-623943 511 bp				
<b>A. implicatus</b> CBS_484.95 aoxB	544bp	Aspimpl scaffold_11 206645-206877 (- strand) 233 bp	phase 2 49 bp	Aspimpl scaffold_11 206305-206595 (- strand) 291 bp	phase 2 61 bp	Aspimpl scaffold_11 205733-206243 (- strand) 511 bp				
<b>A. implicatus</b> CBS_484.95 andB		Aspimpl scaffold_11 207421-207718 298 bp	phase 1 74 bp	Aspimpl scaffold_11 207793-208131 339 bp	phase 1 64 bp	Aspimpl scaffold_11 208196-208473 278 bp	phase 0 58 bp	Aspimpl scaffold_11 208532-209144 613 bp	phase 1 60 bp	Aspimpl scaffold_11 209205-209278 74 bp
<b>A. implicatus</b> CBS_484.95 aoxB2 [paralog]	NA	Aspimpl scaffold_2 49588-49847 (- strand) 260 bp	phase 2 57 bp	Aspimpl scaffold_2 49255-49530 (- strand) 276 bp	phase 2 64 bp	Aspimpl scaffold_2 48680-49190 (- strand) 511 bp				
<b>P. swiecickii</b> 182 6C1 aoxB	613bp	Penswil scaffold_5 470967-471232 266 bp	phase 1 82 bp	Penswil scaffold_5 471315-471605 291 bp	phase 0 60 bp	Penswil scaffold_5 471666-472176 511 bp				
<b>P. swiecickii</b> 182 6C1 andB		Penswil scaffold_5 469712-470354 (- strand) 643 bp	phase 1 62 bp	Penswil scaffold_5 469372-469649 (- strand) 278 bp	phase 0 57 bp	Penswil scaffold_5 468684-469314 (- strand) 631 bp	phase 1 59 bp	Penswil scaffold_5 468551-468624 (- strand) 74 bp		

Footnote: ATG-CAT\* : The column specifies the distance between the start codons of the divergent aoxB and andB genes. NA: Not applicable. The ubiquitous aoxA and andA genes (top of the Table) are never linked. The A. implicatus aoxB2 gene is not linked to a type-II NADH dehydrogenase gene.

**Supplementary Table S2.** NCBI-lodged RNA sequence reads that cover exon-exon fusions in aoxB and andB in *A. calidoustus*, *A. implicatus* and *A. niger* (with full-length aoxB and andB genes).

Species Gene Intron	Database (NCBI)	SRA reads	Species Gene Intron	Database (NCBI)	SRA reads	Species gene Intron	Database (NCBI)	SRA reads
<b><i>A. calidoustus</i> andB intron 1</b> TTAGGCCACT	ERX631993	ERR677102.16649058.2	<b><i>A. calidoustus</i> aoxB intron 1</b> CCAATCTAC	ERX631996	ERR677105.6854148.2			
	ERX631995	ERR677104.15682812.1 ERR677104.15682812.2		ERX631995	ERR677104.2332297.1	<b><i>A. implicatus</i> andB intron 1</b> CTGGCCACT	SRX5208515	not covered
	ERX631996	ERR677105.2162584.1 ERR677105.2162584.2		ERX631994	ERR677103.2510794.2 ERR677103.1656912.1 ERR677103.900396.2	<b><i>A. implicatus</i> andB intron 2</b> TTTGGAAATC	SRX5208515	SRR8399217.21403927.2 SRR8399217.387016.1
<b><i>A. calidoustus</i> andB intron 2</b> TTTGGGATT	ERX631988	ERR677097.373254.2	<b><i>A. calidoustus</i> aoxB intron 2</b> GGATGGATT	ERX631993	ERR677102.2082084.2	<b><i>A. implicatus</i> andB intron 3</b> CCCTCGATTTTA	SRX5208515	not covered
	ERX631990	ERR677099.18680577.2 ERR677099.18680577.1 ERR677099.2869718.1		ERX631992	ERR677101.10577410.1 ERR677101.8218975.1 ERR677101.8193533.1	<b><i>A. implicatus</i> andB intron 4</b> AGTGTCCAG	SRX5208515	SRR8399217.2264778.1 SRR8399217.25258407.1 SRR8399217.992531.1 SRR8399217.23243498.1
	ERX631993	ERR677102.14622834.2						
	ERX631991	ERR677100.9382643.1						
	ERX631996	ERR677105.17252672.2 ERR677105.17252672.1		ERX631996	ERR677105.2013743.2			
<b><i>A. calidoustus</i> andB intron 3</b> CCTCTGTGCTT	ERX631989	ERR677098.13859357.2 ERR677098.2998495.2 ERR677098.2998495.1 ERR677098.22770266.2 ERR677098.13859357.1	<b><i>A. calidoustus</i> aoxB intron 2</b> TATCCCAA	ERX631995	ERR677104.16482873.2 ERR677104.16482873.1	<b><i>A. implicatus</i> aoxB intron 1</b> CCAATCTAT	SRX5208515	SRR8399217.6320403.2
<b><i>A. calidoustus</i> andB intron 4</b> AGTGCCTGG	ERX631992	ERR677101.1542532.2 ERR677101.1542532.1		ERX631994	ERR677103.1971655.2			
	ERX631994	ERR677103.6474623.1 ERR677103.6474623.2		ERX631993	ERR677102.23350232.2 ERR677102.10276109.2 ERR677102.4764038.2 ERR677102.4764038.1	<b><i>A. implicatus</i> aoxB intron 2</b> GGATGGATC	SRX5208515	SRR8399217.23319380.2 SRR8399217.8293776.1 SRR8399217.6320403.1
	ERX631993	ERR677102.17188126.1 ERR677102.17188126.2 ERR677102.12370809.2		ERX631992	ERR677101.7231996.1			
<b><i>A. niger</i> aoxB intron 1</b> CCAATCTAC	SRX9722265	SRR13293334.22709813.2	<b><i>A. niger</i> andB intron 1</b> AGTGCCTAA	SRX11975055	SRR15678655.579521.2	<b><i>A. niger</i> andB intron 3</b> TAGGACAGA	SRX12789281	SRR16587860.3272965.1
<b><i>A. niger</i> aoxB intron 2</b> GGATGGATC	SRX11975789	SRR15679403.6264149.1	<b><i>A. niger</i> andB intron 2</b> TATCCCAA	SRX11975055	SRR15678655.579521.1	<b><i>A. niger</i> andB intron 4</b> CCGAAGCT	SRX18320010	SRR22348904.213785.1
				SRX12789281	SRR16587860.708123.1			
				SRX15688375	SRR19638148.2825246.1			

Footnote: Per database, a maximum of five SRAs covering the exon-exon junction after excision of the indicated intron are given.

Strain gene Intron	Database (NCBI)	SRA reads
<b>A. implicatus</b> <b>aoxB2 intron1</b> CCTG <b>TATA</b> C	SRX5208515	SRR8399217.31224079.1 SRR8399217.30796192.2 SRR8399217.30796192.1 SRR8399217.29964246.2 SRR8399217.29933532.2 SRR8399217.29533741.1
<b>A. implicatus</b> <b>aoxB2 intron2</b> GGGT <b>GCAT</b> C	SRX5208515	SRR8399217.31331017.2 SRR8399217.31167892.1 SRR8399217.30842627.2 SRR8399217.29836882.2 SRR8399217.29657382.2 SRR8399217.29442532.1

**Table S3.** Oligonucleotide primers used for PCR amplification of the *aoxB* and *andB* genes and cDNA prior to sequence verification of mutant alleles.

Strain	Primer	Sequence (5'-)
<i>Aspergillus niger</i> ATCC 1015 <i>aoxB</i> and cDNA	1015-aoxB-5prime-Fw1	CGTTCGATCTTCTCGCTAAG
	1015-aoxB-intern-Rv1	GTGTATAGGTAATGACAGCT
	1015-aoxB-intern-Fw2	GTGCACTGATTCTGTGACTG
	1015-aoxB-3prime-Rv2	CCATAAAGAATAAACACCTGA
	1015-aoxB-5prime-cDNA-Fw1	ATCCCTGCCGAGGTGTCCAA
	1015-aoxB-5prime-cDNA2-Fw1	GAGACAGCTATTGTCTGGAA
	1015-aoxB-intern-cDNA-Rv1	TGACAGCTTCTTCCTCCAGG
<i>Aspergillus niger</i> ATCC 1015 <i>andB</i> gene and cDNA	1015-andB-3prime-Fw1	GCTCGGTACCGAGGAAAGT
	1015-andB-intern-Rv1	GAGCAGAATGACACTGAGCT
	1015-andB-intern-Fw2	GGAAACAGTCTCTGTACATC
	1015-andB-5prime-Rv2	GAACCAGGCCAGATATACAGC
	1015-andB-5prime-cDNA1-Rv2	AGTAGAGGCAGATCCGTCAA
	1015-andB-5prime-cDNA2-Rv2	CCAGGCCAGATATACAGCCTA
<i>Aspergillus niger</i> CBS 554.65 <i>aoxB</i> and <i>andB</i>	1015-andB-3prime-Fw1	GCTCGGTACCGAGGAAAGT
	1015-aoxB-intern-Rv1	GTGTATAGGTAATGACAGCT
	554-65_andB-special-Fw1	GCAAGAGATCCTGGTGAGT
	554-65_andB-special-Fw2	AGTTGCACTGTATCTCGTTG
<i>Aspergillus niger</i> CBS 147343 <i>aoxB</i>	1015-aoxB-5prime-Fw1	CGTTCGATCTTCTCGCTAAG
	554-65_aoxB-intern-Rv1	GTGTATAGGTGATGACAGCT
	1015-aoxB-intern-Fw2	GTGCACTGATTCTGTGACTG
	124-48_transposon-Rv1	CTACAGGGTTACTACCTCAA
	124-48_transposon-Fw1	CCGCCTGTAGCGTTCGAACAA
	1015-aoxB-3prime-Rv2	CCATAAAGAATAAACACCTGA
<i>Aspergillus niger</i> CBS 630.78 <i>aoxB</i>	630-78-aoxB-5prime-Fw1	CGTTTGATCTTCTCGCTAAG
	1015-aoxB-intern-Rv1	GTGTATAGGTAATGACAGCT
	1015-aoxB-intern-Fw2	GTGCACTGATTCTGTGACTG
	630-78-aoxB-3prime-Rw2	CCATAAGAATAAACACCTGAA
<i>Aspergillus niger</i> CBS 769.97 <i>aoxB</i>	630-78-aoxB-5prime-Fw1	CGTTTGATCTTCTCGCTAAG
	1015-aoxB-intern-Rv1	GTGTATAGGTAATGACAGCT
	1015-aoxB-intern-Fw2	GTGCACTGATTCTGTGACTG
	630-78-aoxB-3prime-Rw2	CCATAAGAATAAACACCTGAA