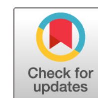
AMERICAN SOCIETY FOR MICROBIOLOGY
Microbiology Resource Announcements

AUTHOR REPLY



Aspergillus Section Flavi, Need for a Robust Taxonomy

Renee S. Arias,[a] Valerie A. Orner,[a] Jaime Martinez-Castillo,[b] Victor S. Sobolev[a]

[a]USDA Agricultural Research Service, National Peanut Research Laboratory, Dawson, Georgia, USA
[b]Unidad de Recursos Naturales, Centro de Investigación Científica de Yucatán A. C., Mérida, Yucatán, Mexico

In a recent letter to the editor, Houbraken et al. (23) provide a series of recommendations to the microbiological community to prevent the taxonomic misidentification of genome-sequenced fungal strains. In the era of genomics and bioinformatics, postulating that 1 nucleotide (nt) within a gene can "correctly" identify a species does not seem plausible. However, the authors of the letter call this the "calmodulin barcode," meaning nucleotide substitutions within a 506-nt region of the calmodulin gene (1). After the evolutionarily conserved rRNA (18S rRNA, internal transcribed spacer [ITS], 28S rRNA) and RNA polymerase II (2–4) showed no differences between *Aspergillus flavus* S- and L-morphotypes, attention shifted toward the calmodulin gene. Thus, without sequencing 18S rRNA, 28S rRNA, or the largest RNA polymerase II subunit, at least 34 new species of *Aspergillus* were named by Houbraken, Frisvad, Visagie, and coworkers (1, 5, 6). However, in a phylogenetic tree of 152 *Aspergillus* section Flavi isolates using the calmodulin 506-nt region, 40 *Aspergillus minisclerotigenes* isolates had only two nucleotide substitutions in common, namely, 100C>A and 269A>G, both of which are silent mutations (Fig. 1). However, only 269A>G discriminates *A. minisclerotigenes* from *A. flavus*, since 100C>A is present in three *A. flavus* isolates (GenBank accession numbers MK451387, MK451365, and MG517986) identified by the authors of the letter. We all agree that species identification is important; paradoxically, the calmodulin barcode assigns species based on a single-nucleotide polymorphism (SNP), while there are between 133,000 and 179,000 SNPs within *A. flavus* S- and L-morphotypes, respectively (7).

Another limitation of *Aspergillus* taxonomy is the chemotypes resulting from 30 genes in the aflatoxin biosynthesis gene cluster (ABC) (8), e.g., *A. flavus* produces B-aflatoxins and *Aspergillus parasiticus* produces B and G types (9). Despite that a single nucleotide change in one ABC gene can prevent aflatoxin production (10), the inheritance of the ABC is favored by environmental pressure (11), and *Aspergillus* spp. are not physically or reproductively isolated; intraspecies and interspecies crosses can result in gain of function, e.g., G-type aflatoxin production (9, 12, 13). Hence, a new species named by one author of the letter was later reversed to its initial name by the same author because of the chemotype, i.e., *A. flavus* S-morphotype to *Aspergillus parvisclerotigenus* (14) and back to *A. flavus* (6). Other groups utilized the calmodulin gene and a single deletion in the ABC to name three new *Aspergillus* species (15, 16).

Analysis of 25 insertion/deletions (indels) within the ABC revealed almost as many *Aspergillus* genotypes as the number of isolates (17, 18). That work was performed not to identify new species but to detect predominant genotypes, sequence their genomes (19, 20), and find targets for gene silencing. Isolates in those publications had been identified by Dr. Bruce Horn comparing morphology to type strains in the culture collection at the National Peanut Research Laboratory, as described (17, 21). Furthermore, SNP analysis of five Ethiopian S-morphotype isolates versus the model genome *A. flavus* NRRL3357 was performed by combining 20,213 nt of evolutionarily conserved genes: rRNA cistron (7,796 nt) and DNA-dependent RNA polymerase largest subunit (RPB1) (5,857 nt) and second largest subunit (RPB2) (6,560 nt). Four S-morphotype isolates had 99.2% identity to *A.*

Editor Antonis Rokas, Vanderbilt University
This is a work of the U.S. Government and is not subject to copyright protection in the United States. Foreign copyrights may apply.
Address correspondence to Renee S. Arias, renee.arias@usda.gov.
This is a response to a letter by Houbraken et al. (https://doi.org/10.1128/MRA.01074-20).
Published 2 December 2021

```
  1  AGCGGAGATG AAGCCGTTGT TATCGCGGTC GAAAACCTTG AAAGCCTCCC GGATCTCCTC CTCAGAGTCG
 71  GTATCCTTCA TCTTTCTCGC CATCATCGTC AGGAACTCTA TTTGAACATT AACAGCTATT TCATTTATGA
                                  A
141  ATCGTACGGA AGCCGTCTCG TACCAGGGAA GTCAATGGTG CCATTGTTGT CGGCGTCAAC CTCGTTAATC
211  ATGTCCTGGA GTTCCGACTC AGAGGGGTTT TGGCCCAGAG AGCGCATGAC AGTGCCCAAC TCCTTGGTGG
                                                                  G
281  TGATCTGGCC TGCATATCAA GTCAATTCAT CAATGCCAAG ACTTCAGCCG ATCGAACAGA AACTGGATAA
351  AATTATGGCG GCTAAAACAT ATTGGTCGCA TTTGAAGGGA GAATGAATAA AATAAACTGT ACTAACCATC
421  ACCGTCCTTG TCCTATAGGA AACCCAAGAA AAGCCAAGTC AGCTTGTGTT CAAATCATGA CGACTAACAA
491  AACCAATTTT CACGAA
```

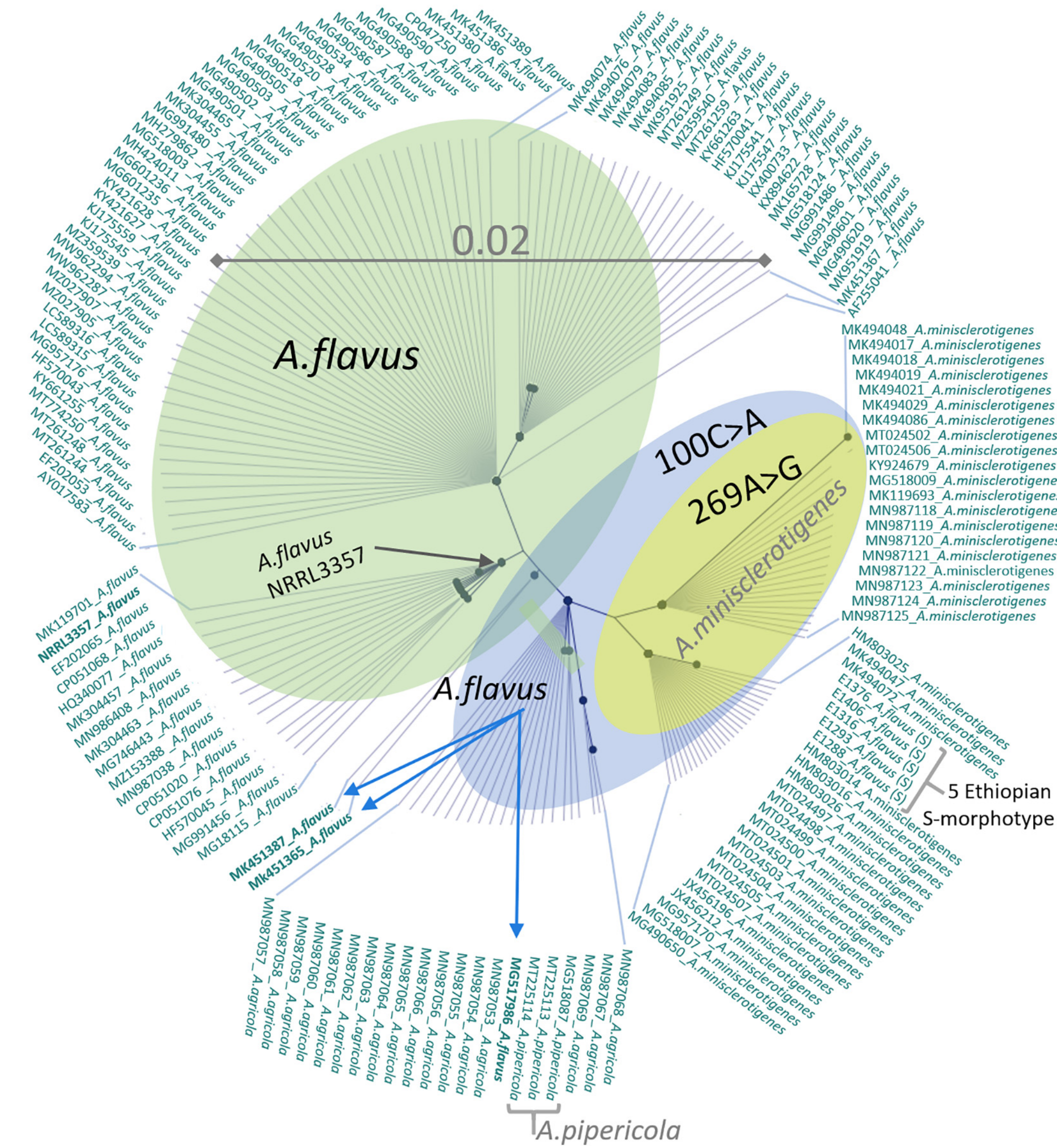


FIG 1 Maximum likelihood phylogenetic tree using 506 nt of the calmodulin gene. (Top) Partial sequence of the calmodulin gene of *Aspergillus*, highlighting the substitutions at positions 100 and 269. (Bottom) Phylogram of 152 isolates of *Aspergillus* species section Flavi, including 87 *A. flavus* isolates (14 SNPs), 40 *A. minisclerotigenes* isolates (10 SNPs), 17 *Aspergillus agricola* isolates (4 SNPs), 3 *Aspergillus pipericola* isolates (2 SNPs), and 5 *A. flavus* S-morphotype isolates (3 SNPs) from Ethiopia; numbers in parentheses correspond to SNPs found within each group. Scale bar, number of substitutions per 100 nt. Blue arrows indicate the 3 *A. flavus* strains with 100C>A substitutions; the yellow area highlights isolates with 269A>G substitutions. After sequence alignment, the neighbor-joining method was used as the construction method with the Jukes-Cantor substitution model including rate variation, processed in CLC Genomics Workbench v.21.0. (Qiagen).

flavus NRRL3357, with SNP detection processed in CLC Genomics Workbench v.21.0 (Qiagen, Denmark). Genome-wide SNP analysis of 24 *Aspergillus* strains, including 13 *A. flavus* isolates (10 L-morphotype and 3 S-morphotype), 10 *Aspergillus oryzae* isolates, and 1 *A. parasiticus*, concluded that, despite their morphological differences, L-type and S-type

isolates have 99.2% identity; therefore, they belong to the same *A. flavus* species (7). The fifth S-morphotype isolate from Ethiopia presented a 189-nt insertion in its rRNA cistron. Unfortunately, the full-length rRNA cistron of *A. minisclerotigenes* is not available for comparison, since it was not reported with its genome (GenBank accession number SWDZ00000000) (22). The current *Aspergillus* taxonomy is based on PCR amplification of short DNA fragments, does not examine the most evolutionarily conserved genes, adopts single indels within the ABC as a classification tool, and observes chemotypes whose detection can vary by orders of magnitude, depending on the analysis methods. New technologies are available, and we think there is a need for an upgrade to a more robust system of classification.

Data availability. The data used to generate the figure are openly available in Harvard Dataverse (https://doi.org/10.7910/DVN/LVJ7ZN).

ACKNOWLEDGMENT

We thank the USDA Department of Agriculture for its financial support, Research Project 6044-21000-005.

REFERENCES

1. Samson RA, Visagie CM, Houbraken J, Hong S-B, Hubka V, Klaassen CHW, Perrone G, Seifert KA, Susca A, Tanney JB, Varga J, Kocsubé S, Szigeti G, Yaguchi T, Frisvad JC. 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. Stud Mycol 78:141–173. https://doi.org/10.1016/j.simyco.2014.07.004.
2. Eickbush TH, Eickbush DG. 2007. Finely orchestrated movements: evolution of the ribosomal RNA genes. Genetics 175:477–485. https://doi.org/10.1534/genetics.107.071399.
3. Yarza P, Yilmaz P, Panzer K, Glöckner FO, Reich M. 2017. A phylogenetic framework for the kingdom Fungi based on 18S rRNA gene sequences. Mar Genomics 36:33–39. https://doi.org/10.1016/j.margen.2017.05.009.
4. Liu YJ, Whelen S, Hall BD. 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. Mol Biol Evol 16:1799–1808. https://doi.org/10.1093/oxfordjournals.molbev.a026092.
5. Pildain MB, Frisvad JC, Vaamonde G, Cabral D, Varga J, Samson RA. 2008. Two novel aflatoxin-producing *Aspergillus* species from Argentinean peanuts. Int J Syst Evol Microbiol 58:725–735. https://doi.org/10.1099/ijs.0.65123-0.
6. Frisvad JC, Hubka V, Ezekiel CN, Hong S-B, Nováková A, Chen AJ, Arzanlou M, Larsen TO, Sklenář F, Mahakarnchanakul W, Samson RA, Houbraken J. 2019. Taxonomy of *Aspergillus* section Flavi and their production of aflatoxins, ochratoxins and other mycotoxins. Stud Mycol 93:1–63. https://doi.org/10.1016/j.simyco.2018.06.001.
7. Chang PK. 2019. Genome-wide nucleotide variation distinguishes *Aspergillus flavus* from *Aspergillus oryzae* and helps to reveal origins of atoxigenic *A. flavus* biocontrol strains. J Appl Microbiol 127:1511–1520. https://doi.org/10.1111/jam.14419.
8. Caceres I, Khoury AA, Khoury RE, Lorber S, Oswald IP, Khoury AE, Atoui A, Puel O, Bailly JD. 2020. Aflatoxin biosynthesis and genetic regulation: a review. Toxins (Basel) 12:150. https://doi.org/10.3390/toxins12030150.
9. Olarte RA, Worthington CJ, Horn BW, Moore GG, Singh R, Monacell JT, Dorner JW, Stone EA, Xie DY, Carbone I. 2015. Enhanced diversity and aflatoxigenicity in interspecific hybrids of *Aspergillus flavus* and *Aspergillus parasiticus*. Mol Ecol 24:1889–1909. https://doi.org/10.1111/mec.13153.
10. Ehrlich KC, Cotty PJ. 2004. An isolate of *Aspergillus flavus* used to reduce aflatoxin contamination in cottonseed has a defective polyketide synthase gene. Appl Microbiol Biotechnol 65:473–478. https://doi.org/10.1007/s00253-004-1670-y.
11. Carbone I, Jakobek JL, Ramirez-Prado JH, Horn BW. 2007. Recombination, balancing selection and adaptive evolution in the aflatoxin gene cluster of *Aspergillus parasiticus*. Mol Ecol 16:4401–4417. https://doi.org/10.1111/j.1365-294X.2007.03464.x.
12. Moore GG, Horn BW, Elliott JL, Hell K, Chulze SN, Barros G, Wright G, Naik MK, Carbone I. 2009. Sexual reproduction influences aflatoxin chemotype diversity in worldwide populations of *Aspergillus flavus* and *A. parasiticus*. Phytopathology 99:S88.
13. Olarte RA, Horn BW, Dorner JW, Monacell JT, Singh R, Stone EA, Carbone I. 2012. Effect of sexual recombination on population diversity in aflatoxin production by *Aspergillus flavus* and evidence for cryptic heterokaryosis. Mol Ecol 21:1453–1476. https://doi.org/10.1111/j.1365-294X.2011.05398.x.
14. Frisvad JC, Skouboe P, Samson RA. 2005. Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B1, sterigmatocystin and 3-*O*-methylsterigmatocystin, *Aspergillus rambellii* sp. nov. Syst Appl Microbiol 28:442–453. https://doi.org/10.1016/j.syapm.2005.02.012.
15. Singh P, Orbach MJ, Cotty PJ. 2018. *Aspergillus texensis*: a novel aflatoxin producer with S morphology from the United States. Toxins 10:513. https://doi.org/10.3390/toxins10120513.
16. Singh P, Callicott KA, Orbach MJ, Cotty PJ. 2020. Molecular analysis of S-morphology aflatoxin producers from the United States reveals previously unknown diversity and two new taxa. Front Microbiol 11:1236. https://doi.org/10.3389/fmicb.2020.01236.
17. Faustinelli PC, Palencia ER, Sobolev VS, Horn BW, Sheppard HT, Lamb MC, Wang XYM, Scheffler BE, Castillo JM, Arias RS. 2017. Study of the genetic diversity of the aflatoxin biosynthesis cluster in *Aspergillus* section Flavi using insertion/deletion markers in peanut seeds from Georgia, USA. Mycologia 109:200–209. https://doi.org/10.1080/00275514.2017.1307095.
18. Mohammed A, Faustinelli PC, Chala A, Dejene M, Fininsa C, Ayalew A, Ojiewo CO, Hoisington DA, Sobolev VS, Martínez-Castillo J, Arias RS. 2021. Genetic fingerprinting and aflatoxin production of *Aspergillus* section Flavi associated with groundnut in eastern Ethiopia. BMC Microbiol 21:239. https://doi.org/10.1186/s12866-021-02290-3.
19. Faustinelli PC, Wang XM, Palencia ER, Arias RS. 2016. Genome sequences of eight *Aspergillus flavus* spp. and one *A. parasiticus* sp., isolated from peanut seeds in Georgia. Genome Announc 4:e00278-16. https://doi.org/10.1128/genomeA.00278-16.
20. Arias RS, Mohammed A, Orner VA, Faustinelli PC, Lamb MC, Sobolev VS. 2020. Sixteen draft genome sequences representing the genetic diversity of *Aspergillus flavus* and *Aspergillus parasiticus* colonizing peanut seeds in Ethiopia. Microbiol Resour Announc 9:e00591-20. https://doi.org/10.1128/MRA.00591-20.
21. Mohammed A, Chala A, Dejene M, Fininsa C, Hoisington DA, Sobolev VS, Arias RS. 2016. *Aspergillus* and aflatoxin in groundnut (*Arachis hypogaea* L.) and groundnut cake in Eastern Ethiopia. Food Addit Contam Part B Surveill 9:290–298. https://doi.org/10.1080/19393210.2016.1216468.
22. Kjærbølling I, Vesth T, Frisvad JC, Nybo JL, Theobald S, Kildgaard S, Petersen TI, Kuo A, Sato A, Lyhne EK, Kogle ME, Wiebenga A, Kun RS, Lubbers RJM, Mäkelä MR, Barry K, Chovatia M, Clum A, Daum C, Haridas S, He G, LaButti K, Lipzen A, Mondo S, Pangilinan J, Riley R, Salamov A, Simmons BA, Magnuson JK, Henrissat B, Mortensen UH, Larsen TO, de Vries RP, Grigoriev IV, Machida M, Baker SE, Andersen MR. 2020. A comparative genomics study of 23 *Aspergillus* species from section Flavi. Nat Commun 11:1106. https://doi.org/10.1038/s41467-019-14051-y.
23. Houbraken J, Visagie CM, Frisvad JC. 2021. Recommendations to prevent taxonomic misidentification of genome-sequenced fungal strains. Microbiol Resour Announc 10:e01074-20. https://doi.org/10.1128/MRA.01074-20.