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Molecular and culture-based identification of *Aspergillus* species in water-impacted homes following Hurricane María in Puerto Rico

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Flooding caused by Hurricane María promoted fungal growth in homes across Puerto Rico, raising concerns about indoor air quality and health risks. This study focuses on identifying *Aspergillus* species from water-impacted homes in San Juan using culture-based and molecular methods. *Aspergillus* is a common indoor contaminant in moisture-damaged environments, with some species associated with significant health risks. However, species-level identification is often limited. To address this, we collected samples from 14 homes, identifying 28 *Aspergillus* isolates through morphological examination and gene sequencing of ITS2, beta-tubulin (*benA*), and calmodulin (*CaM*) genes. Species-level identifications of 22 isolates revealed species belonging to the subgenera *Aspergillus*, *Nidulantes*, and *Circumdanti*. We highlighted the *CaM* gene's importance in molecular identification by phylogenetic analyses, which showed superior resolution in species differentiation. Culture-based methods also played a crucial role in differentiating closely related species, such as *A. flavus* and *A. oryzae*, which molecular methods alone could not reliably separate. Our findings underscore the challenges of *Aspergillus* identification in post-hurricane, water-impacted indoor environments and emphasize the value of integrating phenotypic and genotypic techniques for accurate species identification. These results contribute to a better understanding of fungal composition and its potential public health implications in disaster-affected settings.

Hurricane María, which struck Puerto Rico in September 2017, caused widespread devastation, including severe flooding that damaged homes across the island. The floods created the ideal conditions for indoor fungal growth, prompting air quality concerns and potential respiratory health repercussions for the residents. Our previous research confirmed a significant increase in fungal proliferation inside flooded homes in San Juan, with *Aspergillus* species being the most predominant[1]. This finding aligned with existing literature, which identifies *Aspergillus* species as common fungi in water-damaged environments[2–5].

Aspergillus is a large genus of filamentous fungi within the Ascomycota division, comprising around 350 species[6,7]. The genus is subdivided into six subgenera, 27 sections, and about 75 series, holding significance as both a pathogen and a source of pharmaceuticals, as well as in agriculture and food production[8,9]. Given its medical and economic importance, accurate identification of *Aspergillus* species is essential, as different species vary in pathogenicity and ability to produce harmful mycotoxins. Species-level identification is especially crucial in environments affected by flooding, where *Aspergillus* can pose serious health risks ranging from allergic reactions to severe respiratory infections, depending on the species and the vulnerability of exposed individuals[9–12].

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Isolate	Colony diameter (mm)				CYA 25 °C					MEA 25 °C					CY20S 25 °C				
	CYA 25 °C	MEA 25 °C	CY20S 25 °C	CYA 37 °C	Conidial color	Mycelial color	Reverse color	Soluble pigment	Exudate	Conidial color	Mycelial color	Reverse color	Soluble pigment	Exudate	Conidial color	Mycelial color	Reverse color	Soluble pigment	Exudate
19	24	16.3	28	No Growth	Grey Green	Dull Pink	Brown	Absent	Clear	Grey Green	White to Buff	Orange	Absent	Clear	Grey Green	White	Brown	Absent	Absent
20	38.3	38	42	No Growth	Light Yellow	White to Light Grey	Light Brown	Absent	Absent	Light Yellow	White to Light Grey	Greyish Yellow	Absent	Absent	Light Yellow	White to Light Grey	Light Brown	Absent	Absent
27	38.7	31.3	42	41	Black	White to Dull Yellow	Uncolored	Absent	Clear	Black	White to Dull Yellow	Uncolored	Absent	Clear	Black	White to Dull Yellow	Uncolored	Absent	Clear
33	53	53	58.3	58.7	Black	White to Dull Yellow	Yellow	Absent	Absent	Black	White	Uncolored	Absent	Absent	Black	White to Dull Yellow	Yellow	Absent	Absent
50	11.6	5	10	No Growth	Grey	White	Red Brown	Brown	Absent	Grey	White	Greyish White	Absent	Absent	Grey	White	Yellow	Absent	Absent
73	62	50	47.7	57.3	Black	Pale Yellow	Yellow	Absent	Clear	Black	White to Pale Yellow	White	Absent	Clear	Black	White to Pale Yellow	Pale Yellow	Absent	Absent
75	9	7	9	4	Dull Grey Green	Yellow	Yellow	Absent	Clear	Yellow	Yellow	Yellow	Absent	Clear	Dull Green	Yellow	Orange	Absent	Absent
77	25.6	19	32.6	No Growth	Greyish Blue	White	Pale Brown	Absent	Clear	Greyish Green Blue	White	Uncolored	Absent	Clear	Greyish Blue	White	Orange Brown	Absent	Absent
111	12.7	11	13.5	No Growth	White	White	Brownish Orange	Absent	Absent	White	White	Uncolored	Absent	Absent	White	White	Brownish Orange	Absent	Absent
137	3.7	3.7	7	No Growth	Dull Pale Green	White	Uncolored	Absent	Absent	Dull Pale Green	White	Uncolored	Absent	Absent	Dull Green	White	Pale Grey	Absent	Absent
139	18	16	15	No Growth	Dark Turquoise	White	Orange Brown	Orange Brown	Absent	Greyish Turquoise	White	Uncolored	Yellow	Absent	Greyish Turquoise	White	Uncolored	Absent	Absent
143	0	0	4	No Growth	-	-	-	-	-	-	-	-	-	-	Greyish Turquoise	White	Grey	Absent	-
150	24	13	25	No Growth	Dull Green	Buff	Brown	Absent	Clear	Dull Green	White	Yellow	Absent	Clear	Dull Green	White	Brown	Absent	Absent
155	36	47	55	52.5	Olive	White	Dull Brown	Absent	Absent	Deep Green	White to Yellow	Uncolored	Absent	Absent	Deep Green	White	Dull Brown	Absent	Absent
159	38.3	35.3	42.3	No Growth	Light Yellow	White	Orange Brown	Absent	Absent	Yellow	White	Pale Yellow	Absent	Absent	Yellow	White	Yellow Orange	Absent	Absent
173	3.2	2.3	4.7	No Growth	Pale Green	White	Pale Yellow	Absent	Absent	Greyish Green	White	Uncolored	Absent	Absent	Turquoise Green	White	Pale Yellow	Absent	Absent
215	53.3	31.3	48	No Growth	Light Yellow	White	Dark Brown	Absent	Yellow	Light Yellow	White	Light Brown	Absent	Yellow	Pale Yellow	White	Light Brown	Absent	Absent
226	9	7	9.7	No Growth	Grey Dull Green	Yellow	Yellow	Absent	Absent	Yellow Olive	Yellow	Orange	Absent	Absent	Dull Green	Yellow	Yellow	Absent	Absent
229	42	41	61	51.33	Yellowish Brown	White	Greyish Yellow	Absent	Absent	Yellowish Brown	White	Uncolored	Absent	Absent	Olive Brown	White	Greyish Yellow	Absent	Absent
232	3	2	6.3	No Growth	Dull Grey	White	Uncolored	Absent	Absent	Dull Grey	White	Uncolored	Absent	Absent	Grey	White	Greyish Yellow	Absent	Absent
273	64.3	54.7	64	54	Black	White	Greyish Yellow	Absent	Absent	Black	White	Uncolored	Absent	Absent	Black	White	Yellow	Absent	Absent
276	42.3	37.7	40	41.3	Black	Pale Yellow	Yellow	Absent	Clear	Black	White	Yellow	Absent	Clear	Black	White	Greyish Yellow	Absent	Absent
277	44	40	47.7	48	Black	White	Pale Orange	Absent	Absent	Black	Pale Yellow	Uncolored	Absent	Absent	Black	White	Pale Orange	Absent	Absent

Continued

Isolate	Colony diameter (mm)				CYA 25°C					MEA 25°C					CY20S 25°C				
	CYA 25°C	MEA 25°C	CY20S 25°C	CYA 37°C	Conidial color	Mycelial color	Reverse color	Soluble pigment	Exudate	Conidial color	Mycelial color	Reverse color	Soluble pigment	Exudate	Conidial color	Mycelial color	Reverse color	Soluble pigment	Exudate
293	47.7	36	50	1	Brownish Orange	White	Orange Brown	Absent	Absent	Dull Yellow	White	Uncolored	Absent	Absent	Yellow	White	Pale Orange	Absent	Absent
299	64.6	47.3	40	13.3	Black	White	Greyish Yellow, Black Center	Absent	Clear	Black	White	Uncolored	Absent	Absent	Black	White	Pale Yellow	Absent	Absent
321	12	12.8	16	No Growth	White	Pale Yellow	Pale Yellow	Absent	Absent	White	Pale Yellow	Luteous	Absent	Absent	Yellow Green	Pale Yellow	Pale Orange	Absent	Absent
323	29.3	26.7	25	15.3	Black	White	Pale Yellow	Absent	Clear	Black	White	Pale Orange	Absent	Clear	Black	White	Pale Yellow	Absent	Absent
372	44	46	42.6	50.5	Greyish Yellow	White	Pale Brown	Absent	Clear	Green Yellow	White	Uncolored	Absent	Clear	Olive Yellow	White	Yellow	Absent	Absent
A. fumigatus	18	17.3	21	53.7	Greyish Turquoise	White	Dull Yellow	Absent	Absent	Dark Turquoise	White	Uncolored	Absent	Absent	Greyish Turquoise	White	Dull Yellow	Yellow	Absent

Table 1. Growth rate comparison (mean diameter in mm) and macromorphological characteristics of *Aspergillus* isolates after 7-day incubation in CYA 25 °C, MEA 25 °C, CY20S 25 °C, and CYA 37 °C.

Traditionally, *Aspergillus* classification has relied on macro- and micromorphological features, such as colony diameter, spore color, and microscopic structures like conidial head arrangement and vesicle size[13]. However, identifying closely related species remains challenging and requires specialized training in fungal taxonomy and microscopy[14]. Since the early 2000s, molecular techniques have increasingly complemented morphological approaches, with DNA sequencing becoming a standard tool for species identification[6]. The internal transcribed spacer (ITS) region is the official DNA barcode for fungi. However, secondary markers like calmodulin (*CaM*) and beta-tubulin (*benA*) are often necessary for distinguishing closely related *Aspergillus* species[6,15]. These molecular markers offer greater precision in species differentiation, with amplified sequences typically compared against databases like GenBank[6].

Despite the well-documented role of *Aspergillus* in moisture-damaged indoor environments, research on species-level identification in post-flood conditions remains limited[5,16,17]. Following extreme weather events such as hurricanes, there is a critical need for studies integrating phenotypic and genotypic techniques to accurately identify *Aspergillus* species in water-impacted homes. This work addresses that gap by using culture-based and molecular methods to characterize *Aspergillus* composition in post-Hurricane María homes in San Juan, Puerto Rico. By combining traditional morphological and molecular approaches, it offers a more comprehensive view of fungal presence and species composition in settings susceptible to flooding. This study enhances our understanding of *Aspergillus* species in water-impacted environments and demonstrates the value of integrating both phenotypic and genotypic methods to improve species identification accuracy. Accurate identification is essential for assessing health risks and informing public health interventions to mitigate fungal contamination in flood-affected areas.

Results
Morphological characteristics of *Aspergillus* isolates
We selected 28 *Aspergillus* isolates from the sampled homes and two positive controls (*A. fumigatus* isolate and *A. brasiliensis* genomic DNA) to achieve species-level identification. The isolates were obtained from 13 different homes (classified as water-impacted, including both flooded and rain-infiltrated homes), with most samples collected approximately one year after the hurricane. Isolates were taken from various locations within the homes (seven from living rooms, seven from kitchens, six from bedrooms, six from bathrooms, and two from outdoor areas). Six isolates originated from sites with > 1000 CFU/m³ total *Aspergillus* spp., and eighteen from sites where *Aspergillus* spp. accounted for > 50% of total filamentous fungi (Supplementary Table S1). To provide a culture-based identification to the species level of the *Aspergillus* isolates selected, we collected a comprehensive description of macro and micromorphological characteristics applicable to the identification of common *Aspergillus* species (Tables 1 and 2). Colony growth rates on various standardized media and microscopical characteristics are taxonomically informative (Fig. 1). Through the culture-based approach and using three different identification keys (Klich, Klich & Pitt, Samson), we were able to identify 22 out of 28 of our *Aspergillus* isolates and the positive control (*A. fumigatus*) (Table 2). The identified isolates belong to different subgenera (*Aspergillus*, *Circumdanti,* and *Nidulantes*). Isolates A50, A77, A111, A143, A159, A293 and A299 remained unidentified.

DNA extraction and polymerase chain reaction (PCR) of *Aspergillus* isolates obtained
To begin the molecular identification approach, we successfully extracted the genomic DNA of all 28 *Aspergillus* isolates and positive control (*A. fumigatus*). The genomic DNA concentration from our samples ranged from 11 to 120 ng/μL; 50 μL of the product was used for PCR amplification experiments. To achieve fungal identification to the species level of *Aspergillus* isolates, we amplified the internal transcribed spacer region 2 (ITS2), beta-tubulin (*benA*), and calmodulin (*CaM*) target genes via PCR. Twenty-two out of twenty-eight *Aspergillus* isolates were positive to PCR amplification of ITS2, *benA*, and *caM* genes with amplicon sizes ranging between 300–400 base pairs (bps), 400–5660 bps, and 400–580 bps, respectively. Annealing temperature troubleshooting results for *Aspergillus* isolates A27, A73, A273, A276, and A299 were unsuccessful.

Gene sequence homology of Aspergillus isolates
We employed Basic Logical Alignment Search Tool (BLAST) results from the ITS2, *benA*, and *CaM* gene sequences of this study on the National Center for Biotechnology Information (NCBI) to assess their sequence similarity with reference sequences in GenBank. The results in Table 3 revealed that most isolates had an identity above 99% similar to reference sequences in GenBank.

Comparison between culture and molecular-based methods for Aspergillus isolates identification
Results from culture-based and molecular-based approaches matched for eleven (11) out of the twenty-eight (28) *Aspergillus* isolates compared (isolates A19, A33, A75, A137, A139, A150, A155, A215, A226, and A229) and the positive control (*A. fumigatus*). The culture-based approach provided preliminary species identification to isolates A27, A73, A273, A276, and A277, which couldn't be identified through molecular-based approaches (no PCR products were obtained). More importantly, macro and micromorphological characteristics were very useful in distinguishing the closely related species *A. flavus* and *A. oryzae* (isolates A155 and A372), where ITS2, *benA*, and *CaM* genes showed > 100% similarity to both *A. flavus* and *A. oryzae*. Although culture-based techniques yielded incorrect species identification to five isolates (A20, A173, A232, 321, and 323), the species identified were classified under the same subgenera and/or section (Supplementary Table S2). For example, isolate A20 (*A. steynii*) was incorrectly classified as *A. westerdijkiae* through culture techniques, but both species belong to the *Circumdanti* subgenera. The same is observed for isolate A323 (*A. brunneoviolaceus*), identified as *A. japonicus* through the culture approach, but both species are members of the *Nigri* section. Isolates A173 (*A. gracilis*) and A232 (*A. hordei*) are both incorrectly classified as *A. penicillioides* through culture-based techniques,

Isolate	Conidial heads	Seriation	Vesicle shape	Vesicle size	Conidial shape	Conidial size	Conidial texture	Identity of isolates
19	Radiate	Biseriate	Pyriform	8 μm	Globose	3.24 μm	Smooth	*A. versicolor*
20	Radiate	Biseriate	Globose	47 μm	Globose	2.65 μm	Smooth	*A. westerdijkiae*
27	Radiate	Biseriate	Globose	81 μm	Globose	4.11 μm	Finely Roughened	*A. foetidus*
33	Radiate	Biseriate	Globose	61 μm	Globose	4.5 μm	Rough	*A. niger*
50	Radiate	Biseriate	Globose	7.99 μm	Globose	2.23 μm	Rough	Unidentified
73	Radiate	Biseriate	Globose	34.10 μm	Globose	2.04 μm	Finely Roughened	*A. niger var. awamori*
75	Radiate	Uniseriate	Globose	33.49 μm	Spherical and Ovoid	5.41 × 4 μm	Spinose	*Eurotium chevalieri = A. chevalieri*
77	Radiate	Biseriate	Sub globose	9.5 μm	Globose	2.56 μm	Smooth	Unidentified
111	Radiate	Biseriate	Globose	7.28 μm	Globose	3.69 μm	Smooth	Unidentified
137	Radiate	Uniseriate	Spathulate	10.82 μm	Subspherical	4.01 μm	Finely Roughened	*A. penicillioides*
139	Radiate	Biseriate	Sub globose	10.84 μm	Spherical	3.56 μm	Rough	*A. sydowii*
143	Radiate	Uniseriate	Pyriform	11.35 μm	Globose	3.25 μm	Smooth	Unidentified
150	Radiate	Biseriate	Spathulate	12.57 μm	Globose	2.9 μm	Finely Roughened	*A. versicolor*
155	Radiate to Columnar	Uniseriate	Elongate	12.69 μm	Globose	6.79 μm	Finely Roughened	*A. flavus*
159	Radiate	Biseriate	Globose	36.17 μm	Globose	2.12 μm	Smooth	Unidentified
173	Loosely Columnar	Uniseriate	Spatulate	7.63 μm	Globose	2.72 μm	Smooth	*A. penicillioides*
215	Radiate	Biseriate	Globose	29.81 μm	Globose	3.5 μm	Finely Roughened	*A. westerdijkiae*
226	Radiate	Uniseriate	Globose	25 μm	Ovoid	4.6 μm	Finely Roughened	*Eurotium chevalieri*
229	Radiate	Both	Globose	6.88 μm	Globose	5.0 μm	Coarsely Roughened	*A. tamarii*
232	Radiate	Uniseriate	Pyriform	10.65 μm	Globose	2.16 μm	Smooth	*A. penicillioides*
273	Radiate	Biseriate	Globose	55.87 μm	Globose	3 μm	Smooth	*A. awamori*
276	Radiate	Uniseriate	Globose	55.56 μm	Globose	4.05 μm	Coarsely Roughened	*A. niger var. niger*
277	Radiate	Uniseriate	Globose	76.87 μm	Globose	3.83 μm	Rough	*A. aculeatus*
293	Radiate	Biseriate	Globose	13.29 μm	Globose	1.24 μm	Smooth	Unidentified
299	Radiate	Uniseriate	Globose	54.6 μm		3.82 μm	Smooth	Unidentified
321	Radiate	Uniseriate	Sub globose	30.6 μm	Sub globose	7.6 μm	Finely Roughened	*Eurotium rubrum*
323	Radiate	Uniseriate	Globose	31.1 μm	Globose	4.42 μm	Rough	*A. japonicus*
372	Radiate	Uniseriate	Globose	18.56 μm	Globose	4.01 μm	Finely Roughened	*A. oryzae*
A. fumigatus	Columnar	Uniseriate	Spathulate	17.18 μm	Ovoid	2 μm	Smooth	*A. fumigatus*

Table 2. Micromorphological characteristics of *Aspergillus* isolates after G25N microcultures observation.

but they all belong to the *Restricti* section. In terms of isolate A321 (*A. pseudoglaucus*), which was incorrectly identified through macromorphological characterization as *A. rubrum* (formerly *Eurotium rubrum*), a note was highlighted in the identification key stating the close resemblance of this species to *Eurotium repens*, now known as *A. pseudoglaucus*.

Phylogenetic analysis of *Aspergillus* isolates

We inferred the evolutionary history of the *Aspergillus* species isolated from the sampled homes using the Maximum Likelihood method and the Tamura-Nei model in MEGA12[18]. To measure the consistency of the phylogenetic tree, we employed a bootstrap of 1000 replications. The percentage of trees in which the associated taxa clustered is shown next to the branches. Sequences generated in this study are marked with a red diamond in the phylogenetic tree, while reference sequences obtained from GenBank are unmarked. *Hamigera avellanea* (*Aspergillaceae*) was used as the outgroup in all trees. The phylogenetic analysis of *Aspergillus* isolates showed that they grouped into distinct clusters, indicating close genetic relationships.

The phylogenetic tree based on the ITS2 gene revealed that the alignment matrix contained 70 sequences, comprising 28 isolates from this study and 42 representative sequences from GenBank (Supplementary Table S3). After applying a 95% site coverage threshold to exclude positions with gaps or missing data in more than 5% of sequences, the final dataset consisted of 242 nucleotide positions. Isolates from *Aspergillus* species classified under the same taxonomical section had above 83% bootstrap support, with several species-level clusters reaching 99% (e.g., *A. versicolor*, *A. sydowii*, *A. westerdijkiae*) (Fig. 2).

The beta-tubulin (*benA*) gene alignment matrix contained 69 sequences (28 isolates from this study and 41 reference sequences from GenBank). To reduce the influence of poorly aligned regions, the partial deletion option was applied, excluding positions with less than 95% site coverage, resulting in a final dataset of 277 positions. All isolates showed > 83% bootstrap-supported clustering with known reference species from GenBank (Fig. 3). High-confidence clusters were observed for species such as *A. versicolor*, *A. sydowii*, and the closely related pair *A. flocculosus* / *A. ochraceopetaliformis*, each receiving 99–100% support. In contrast, some members of the *Nigri* section showed weaker support, ranging from 57–70%.

The phylogenetic tree based on the *CaM* gene had an alignment matrix that also included 70 sequences (28 isolates from this study and 42 reference sequences from GenBank). The partial deletion option was applied to

| MEA 25°C | CYA 25°C | CY20S 25°C | CYA 37°C | Microscopy (60X) |
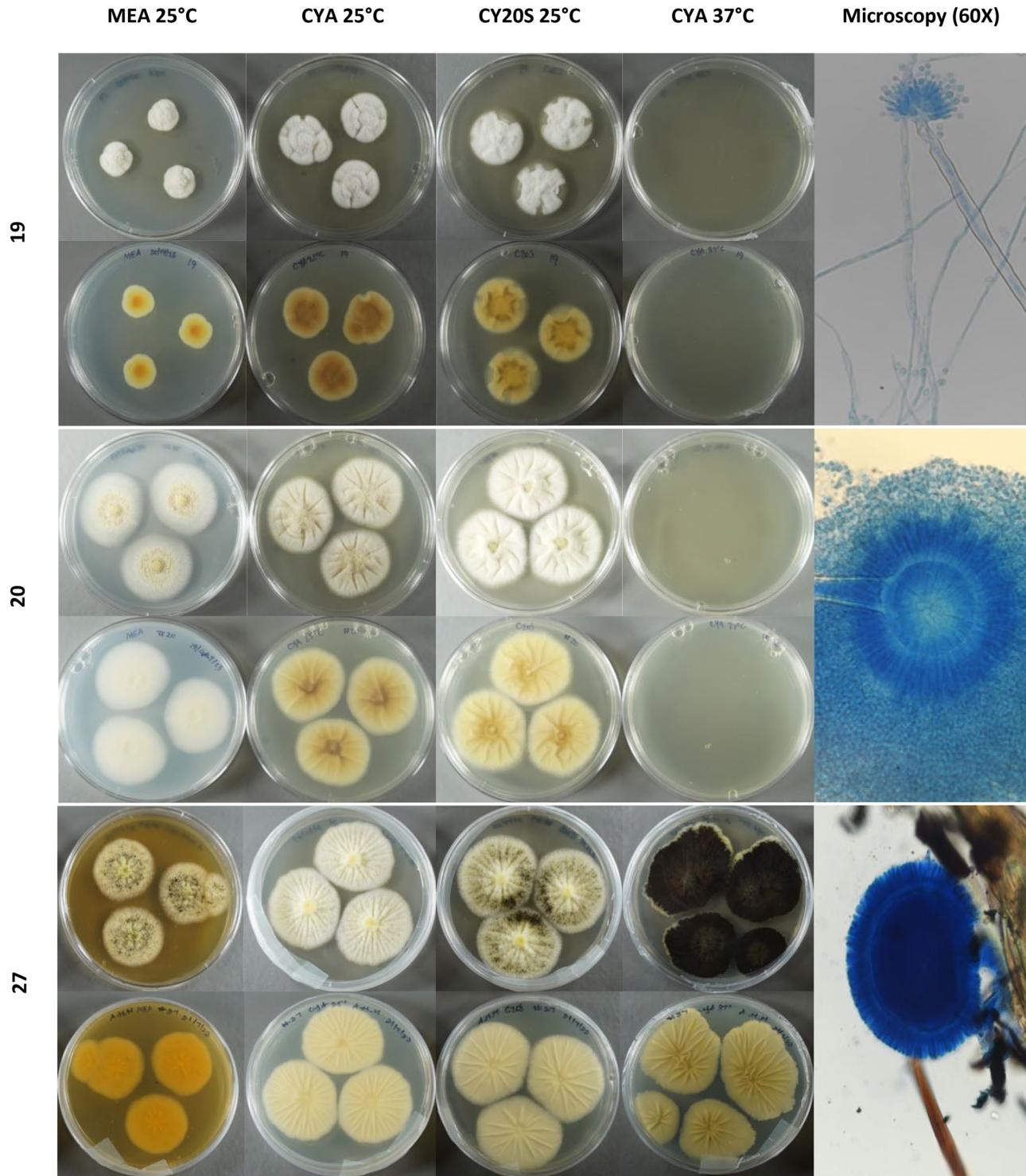
Fig. 1. Macro and microscopic growth of *Aspergillus* isolates. Macroscopic growth comparison, from left to right, on MEA, CYA, CY20S (25 °C) and CYA (37 °C), after 7-day incubation. Microscopic photos from G25N microcultures on 60X.

eliminate positions with less than 95% site coverage, resulting in a final alignment of 366 positions. All isolates, except for *A. petersonii*, showed over 80% cluster similarities with representative sequences from GenBank (Fig. 4). Different but closely related species like *A. flocculosus/A. ochraceopetaliformis* (series *Steynorium*) and *A. flavus/A. oryzae* (series *Flavi*) were still clustered together in their respective clades.

MEA 25°C	CYA 25°C	CY20S 25°C	CYA 37°C	Microscopy (60X)
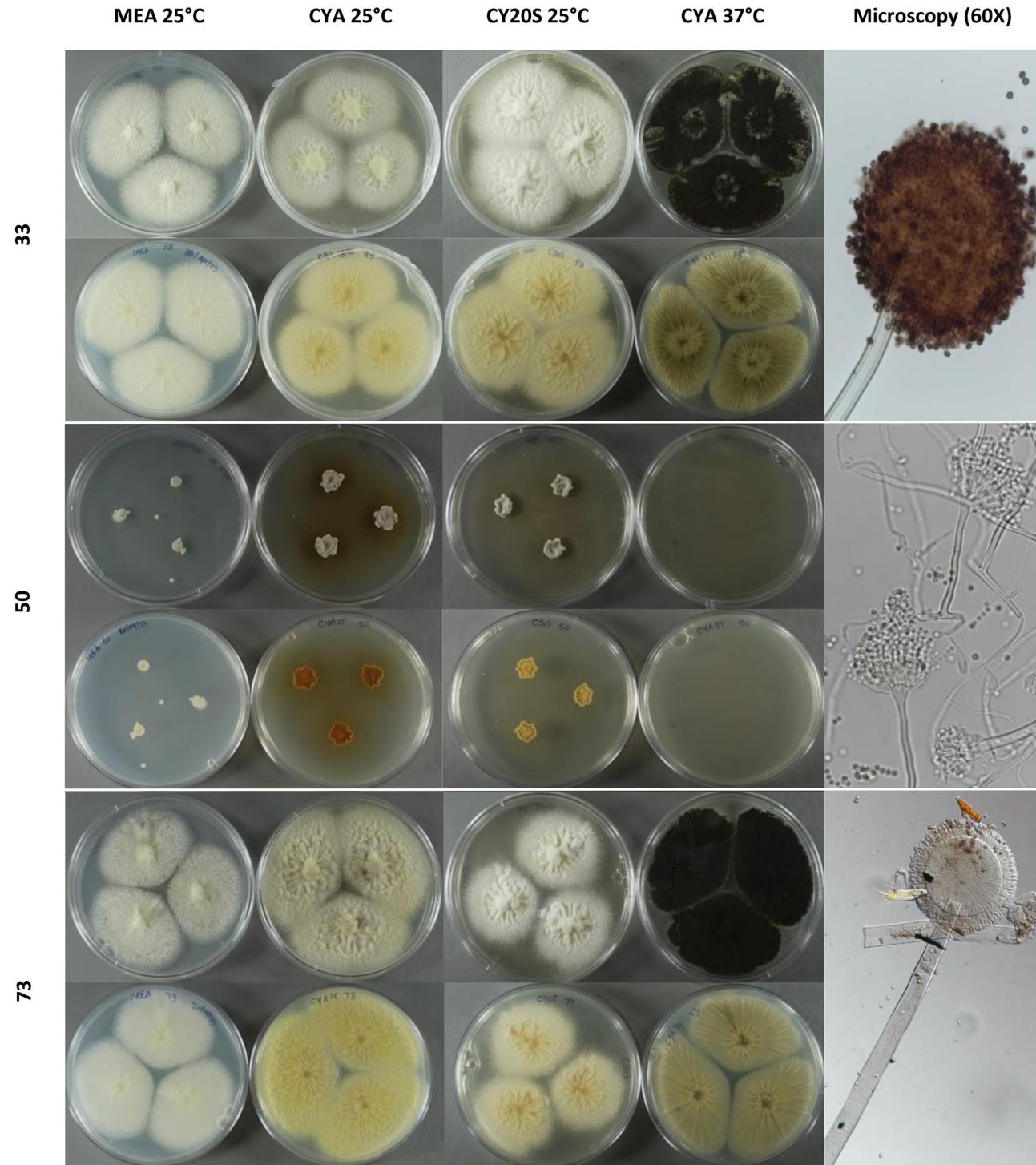
Fig. 1. (continued)

Discussion

In this study, we applied both phenotypic and genotypic methods to achieve species-level identification of 28 *Aspergillus* isolates from homes affected by Hurricane María in San Juan, Puerto Rico. Using molecular techniques, we identified 22 isolates at the species level, categorizing them into the subgenera *Circumdanti* (n = 12), *Aspergillus* (n = 8), and *Nidulantes* (n = 3). The *Circumdanti* and *Nidulantes* subgenera, each encompassing over 100 species, represent some of the most diverse *Aspergillus species*[19,20]. Meanwhile, the *Aspergillus* subgenus, characterized by xerophilic species, thrives in low-moisture environments, making it prevalent indoors[21,22]. Beyond identification, documenting the variety of *Aspergillus* species in post-hurricane homes provides a regional baseline for Puerto Rico that can support environmental health surveillance and future longitudinal
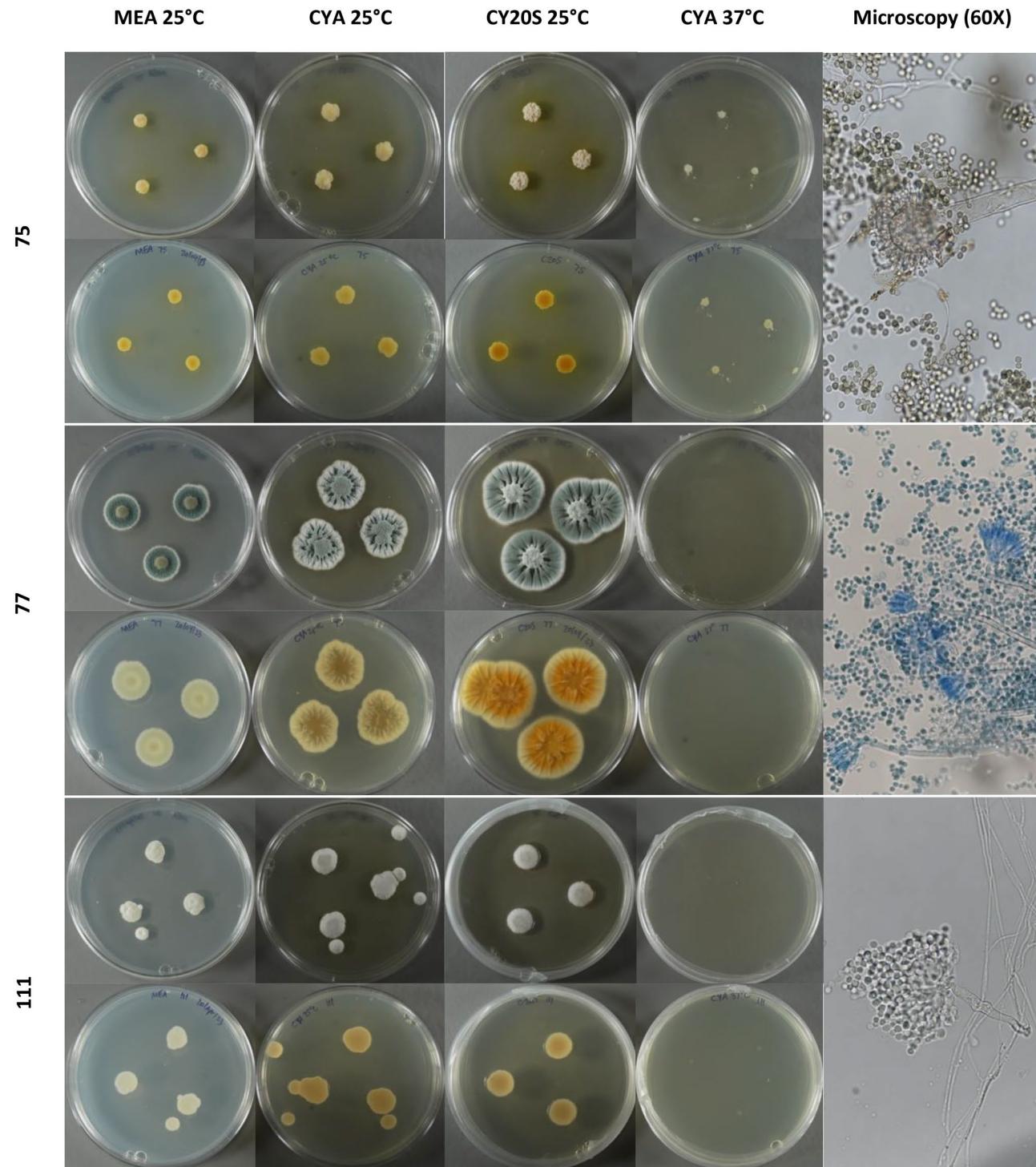
Fig. 1. (continued)

analyses to track fungal populations over time. This information is particularly important in the Caribbean, where post-disaster mycological studies are scarce and the warm/humid climate supports the growth of fungi. Moreover, because different *Aspergillus* species may have varying levels of pathogenic potential[23], species-level identification can help assess public health risks and better prepare for future hurricane events.

Our phylogenetic analysis of ITS2, *benA*, and *CaM* sequences showed distinct clustering patterns. ITS2-based trees showed strong bootstrap support (≥83%) for most section-level groupings, with species like *A. versicolor*, *A. sydowii*, and *A. westerdijkiae* forming distinct clades supported at 99–100%. The *benA* gene tree also produced high-confidence clusters, particularly for *A. versicolor*, *A. sydowii*, and the pair *A. flocculosus / A. ochraceopetaliformis*, though bootstrap support was lower (57–70%) for some *Nigri* section members. The
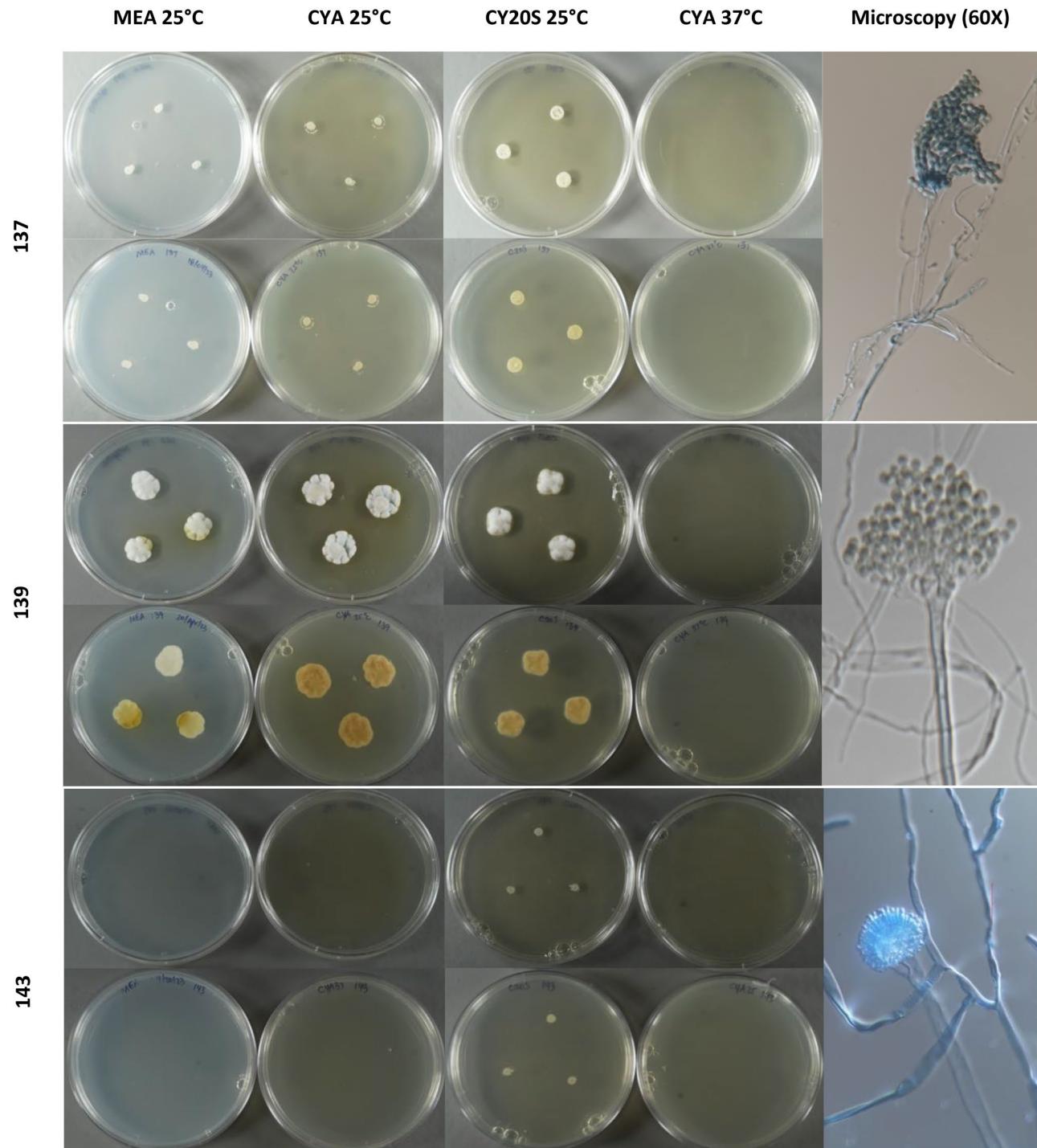
Fig. 1. (continued)

CaM gene tree resolved most isolates at the species level, except for *A. petersonii*, with species-level clusters for *A. flavus*, *A. oryzae*, and *A. westerdijkiae* supported at 98–100%. These results reinforce the *CaM* gene's superior discriminatory power compared to ITS2 and *benA*, consistent with previous studies[24,25].

However, six isolates, likely belonging to the black *Aspergilli* in the *Nigri* section, could not be identified at the species level due to the limitations of both molecular and phenotypic methods[26,27]. While sequence-based identification is the gold standard, morphological traits remain essential for resolving closely related species. For example, in this study, differentiating *A. flavus* from *A. oryzae* required morphological analysis, as molecular results alone were inconclusive[28]. Additionally, morphology-based methods allowed for preliminary identification of five black *Aspergilli* isolates that molecular methods could not identify, underscoring the complementary role of culture techniques in complex identifications[29]. Despite identifying 22 isolates through

| MEA 25°C | CYA 25°C | CY20S 25°C | CYA 37°C | Microscopy (60X) |
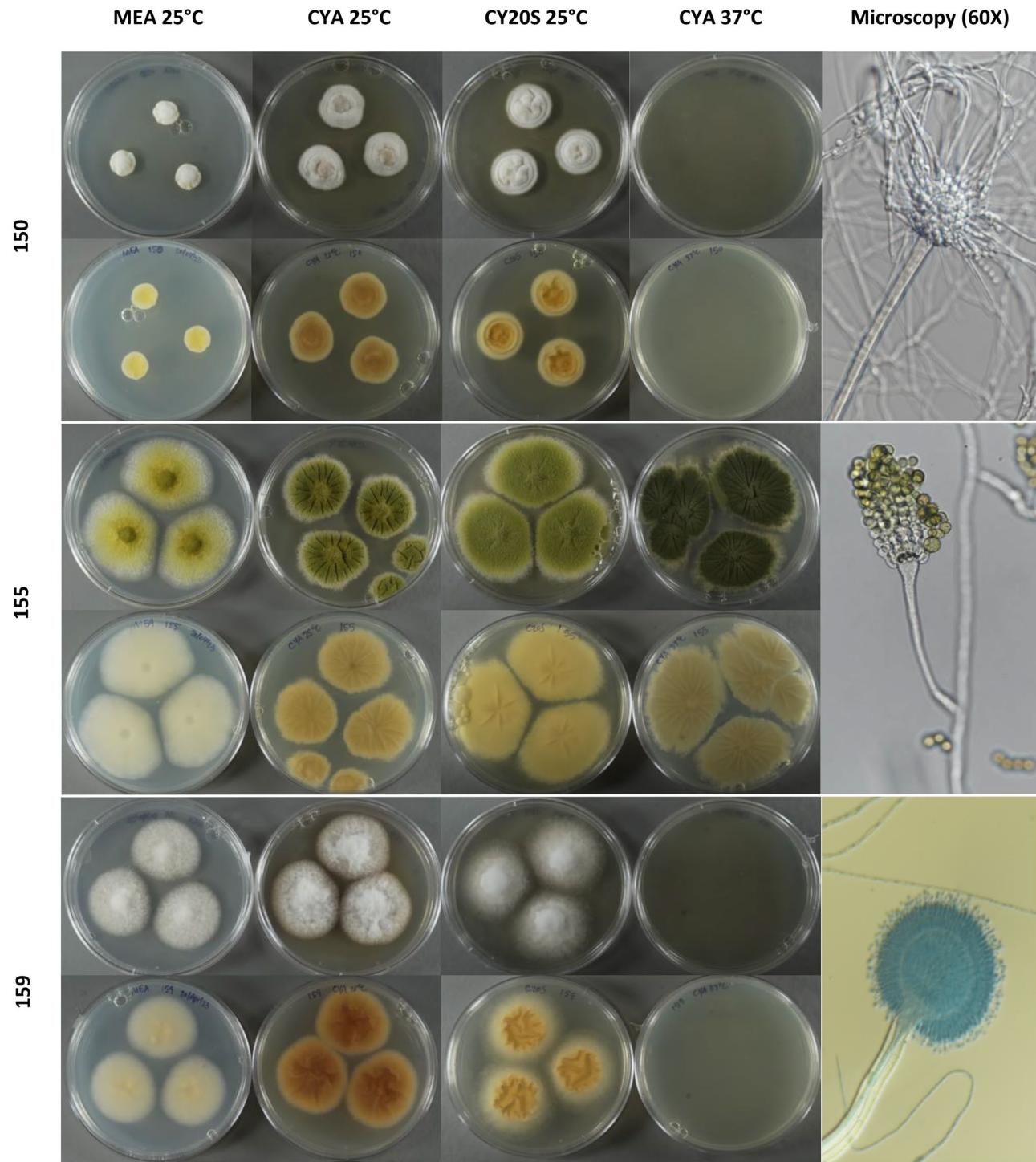
Fig. 1. (continued)

morphological keys, five showed discrepancies with molecular results but remained in the same subgenus or section, highlighting limitations of morphological identification for certain taxonomic groups[30]. Expanding morphological keys is critical for identifying emerging *Aspergillus* species in indoor air environments.

Most isolates identified in this study, such as *A. flavus* (A155), *A. niger* (A33), *A. penicillioides* (A137), *A. sydowii* (A139), *A. tamarii* (A229), *A. versicolor* (A19, A150), and *A. westerdijkiae* (A215), have previously been reported as common indoor fungal contaminants[19,31]. Specifically, *A. versicolor*, *A. sydowii*, and *A. penicillioides*, all from xerophilic or primary colonizer sections, are often associated with building dust and are well adapted to indoor environments[21,22]. In Puerto Rico, these species have been detected at high concentrations in indoor fungal population studies[32,33]. Taxonomic classification of our isolates revealed representation across sections, including *Circumdanti*, *Nigri*, *Petersoniorum*, *Candidi*, and *Flavi*. For example, isolates from section

| MEA 25°C | CYA 25°C | CY20S 25°C | CYA 37°C | Microscopy (60X) |
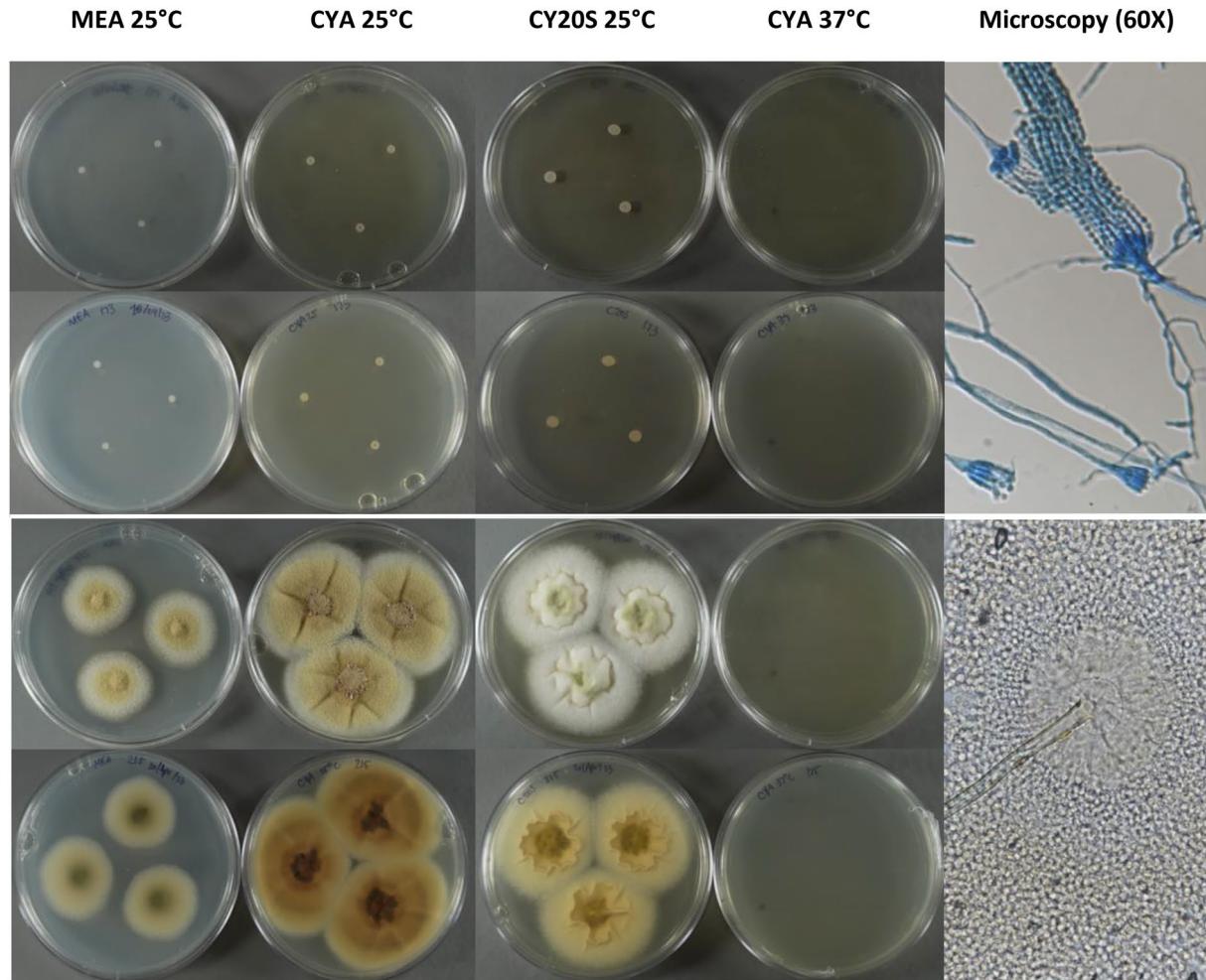
Fig. 1. (continued)

Circumdanti, such as *A. steynii* (A20) and *A. westerdijkiae* (A215), are known producers of the mycotoxin ochratoxin A[34]. Members of the *Nigri* section, such as *A. niger* and *A. brunneoviolaceus*, are also prevalent indoor contaminants[35,36]. Finally, *Flavi* section members like *A. flavus* and *A. oryzae* highlight the health implications of mycotoxigenic *Aspergillus* species in indoor air[37]. While many of these species are recognized as typical indoor fungi, their frequent recovery in this study suggests they may become more dominant or persistent in post-flood indoor environments, particularly under the warm and humid conditions typical of Puerto Rico. Though *Aspergillus* species have been broadly reported after hurricanes[1,2,5,38,39], this study provides one of the few species-level analyses of indoor *Aspergillus* communities in a post-hurricane setting[4,17,40]. Our findings highlight *A. versicolor*, *A. flavus*, *A. niger*, *A. westerdijkiae*, and *A. sydowii* as prevalent species in water-impacted homes, warranting further study into their environmental persistence and potential health risks.

The species we detected are consistent with organisms that proliferate in house dust and can contribute to adverse respiratory outcomes[31,41]. Moisture intrusion and damaged building materials sustain colonization and elevate airborne spores, which are linked to upper- and lower-airway symptoms and asthma exacerbations[42–44]. Beyond air, *Aspergillus* can persist in plumbing biofilms and building water systems and be released into the air during activities like showering, providing an indoor exposure route that is especially relevant after flooding[45,46]. Several of the species we recovered (e.g., *A. versicolor*, *A. flavus*, *A. niger*, *A. westerdijkiae*, *A. sydowii*) are common in indoor dust[41]; some are also toxigenic (e.g., *A. versicolor* → sterigmatocystin; *A. westerdijkiae* → ochratoxin A, indole alkaloids), underscoring potential combined allergenic, infectious, proinflammatory and toxic exposures in water-impacted homes[47,48]. Although *A. fumigatus* was not isolated in our set (included only as a control), sensitization to *A. fumigatus* is a recognized severe-asthma phenotype associated with worse airflow obstruction, higher treatment requirements, and structural airway disease, reinforcing the broader clinical importance of *Aspergillus* exposures in post-disaster settings[49]. Hot, humid conditions such as those in the Caribbean favor indoor fungal growth. Across studies from varied geographies, indoor *Aspergillus* exposure is associated with allergic rhinitis and asthma symptoms/exacerbations[50,51]; among susceptible hosts, it is linked to allergic bronchopulmonary aspergillosis (ABPA) and chronic pulmonary aspergillosis (CPA)[23], and invasive aspergillosis remains a risk for immunocompromised individuals[44,52]. Non-pulmonary diseases relevant to the region include otomycosis (frequently due to *A. niger* and related species) and fungal keratitis (often involving
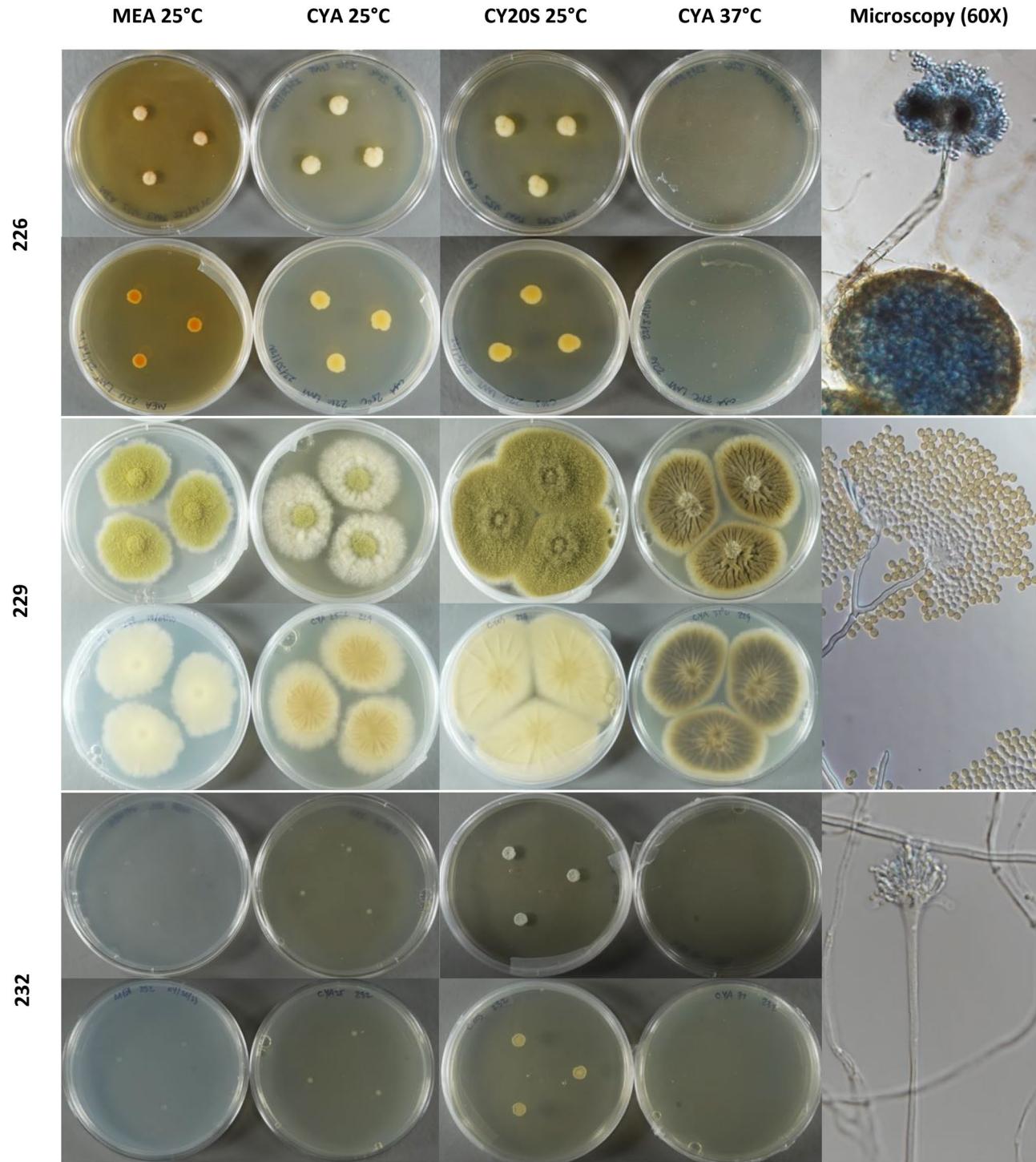
Fig. 1. (continued)

A. flavus in tropical climates)[53,54]; cutaneous/wound aspergillosis can occur where the skin barrier is disrupted after disasters[55]. Complementing these exposure–response patterns, recent work in Puerto Rican infants born after Hurricane María showed nasal mycobiome dysbiosis within the first year post-hurricane, indicating the potential of disaster-related fungal exposures to shape early-life airways[56].

The growth of indoor *Aspergillus* spp. and other filamentous fungi is best controlled by eliminating and removing moisture sources. Key measures include: (i) fixing leaks and venting moisture-generating appliances to the outdoors[57–59]; (ii) drying all wet materials within 24–48 hours[58,59]; (iii) discarding porous items that remained wet (e.g., gypsum board, carpets, furniture)[58,59]; (iv) cleaning hard, non-porous surfaces with detergent and using a High-Efficiency Particulate Air (HEPA)-vacuum after drying[58]; and (v) keeping the indoor relative humidity below ~ 60% (ideally 30–50%) via dehumidification and improved ventilation[59]. Given the clinical relevance of
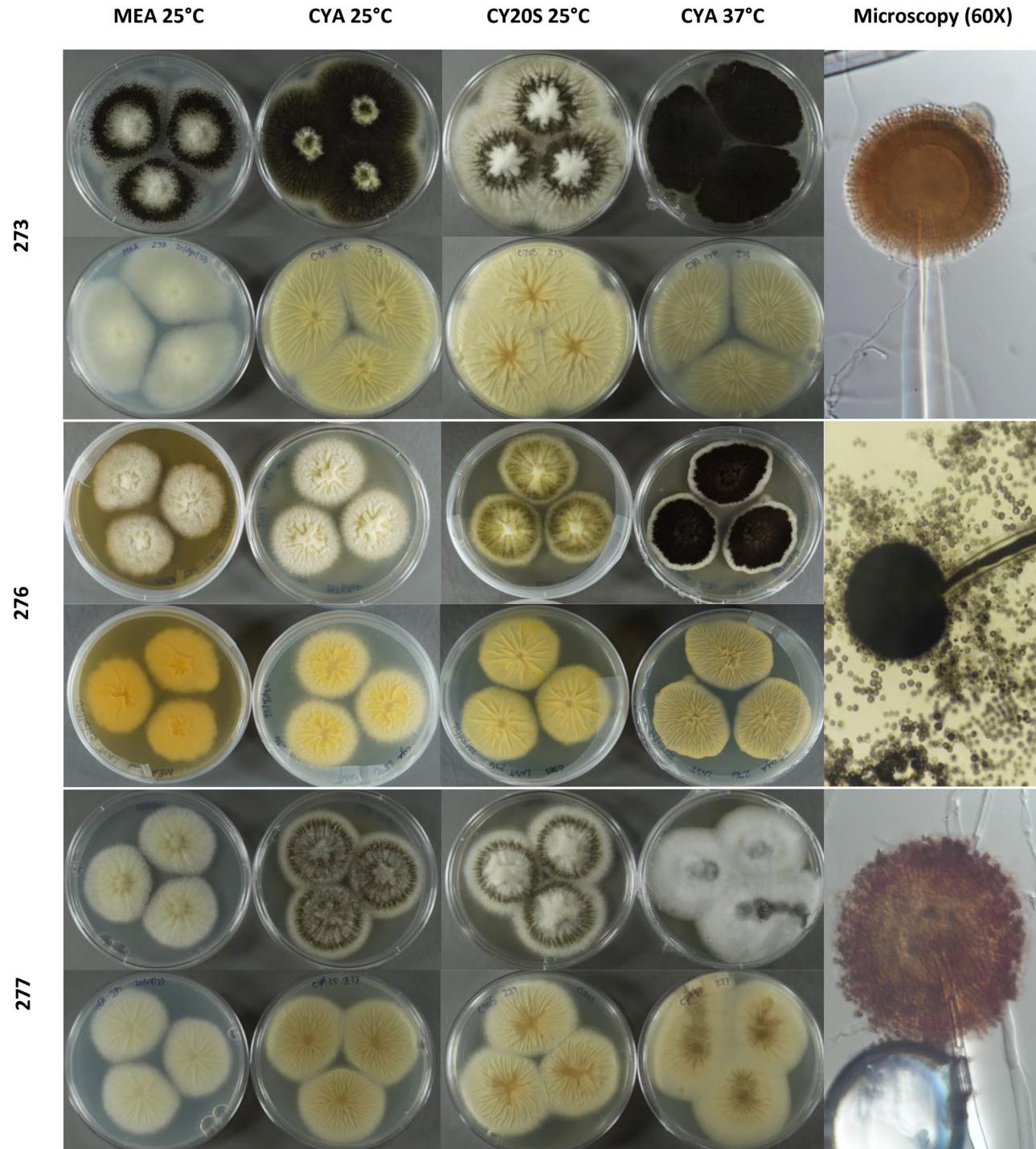
Fig. 1. (continued)

Aspergillus in asthma and allergy, remediation should also protect occupants and workers with at least an N95 respirator, eye protection, and gloves during debris removal and cleaning[58]. Additionally, the use of portable HEPA filter air purifiers in occupied rooms helps to reduce airborne spore levels in the air[59,60]. Consistent with our prior work in naturally ventilated Puerto Rican homes, outdoor fungal levels significantly influenced indoor levels, highlighting an outdoor–indoor air continuum after disasters[1]. Control must therefore extend beyond the building envelope: prompt removal of organic debris (fallen branches, wood piles, water-damaged materials) from yards, sidewalks, and immediate surroundings, along with community-scale debris pickup, can reduce neighborhood reservoirs of filamentous fungi that otherwise re-enter homes through open windows and doors.

 This study has several strengths, notably the combined use of phenotypic and genotypic techniques for *Aspergillus* identification. The integration of morphological methods with molecular analysis using ITS2,
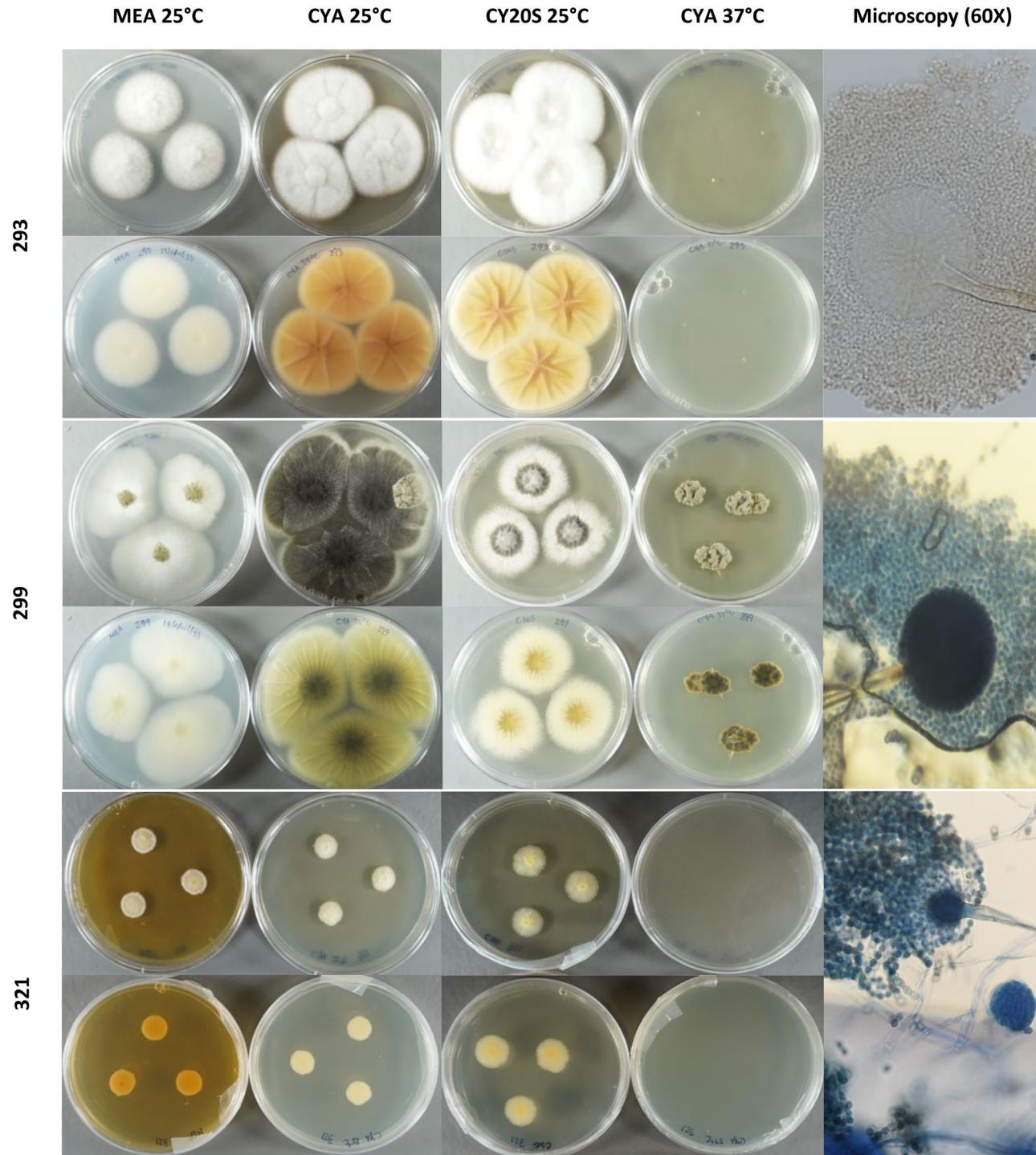
Fig. 1. (continued)

ß-tubulin (*benA*), and especially the calmodulin (*CaM*) gene, which provided superior resolution for species differentiation, allowed for more accurate species-level identification and phylogenetic analysis of *Aspergillus* isolates. Morphological characterization using culture techniques also played a key role in distinguishing closely related species like *A. flavus* and *A. oryzae*, which were difficult to tell apart using molecular methods alone[28]. This combined strategy is especially important in post-flood indoor settings, where precise identification can help guide effective public health responses.

However, there are limitations to consider. Molecular techniques, while powerful, did not fully resolve all isolates, especially black *Aspergilli* in the *Nigri* section, where species-specific differentiation remains challenging[27,61]. Additionally, the final datasets used in our Maximum Likelihood trees were reduced through partial deletion (to 95% site coverage), resulting in 242, 277, and 366 nucleotide positions for ITS2, *benA*, and
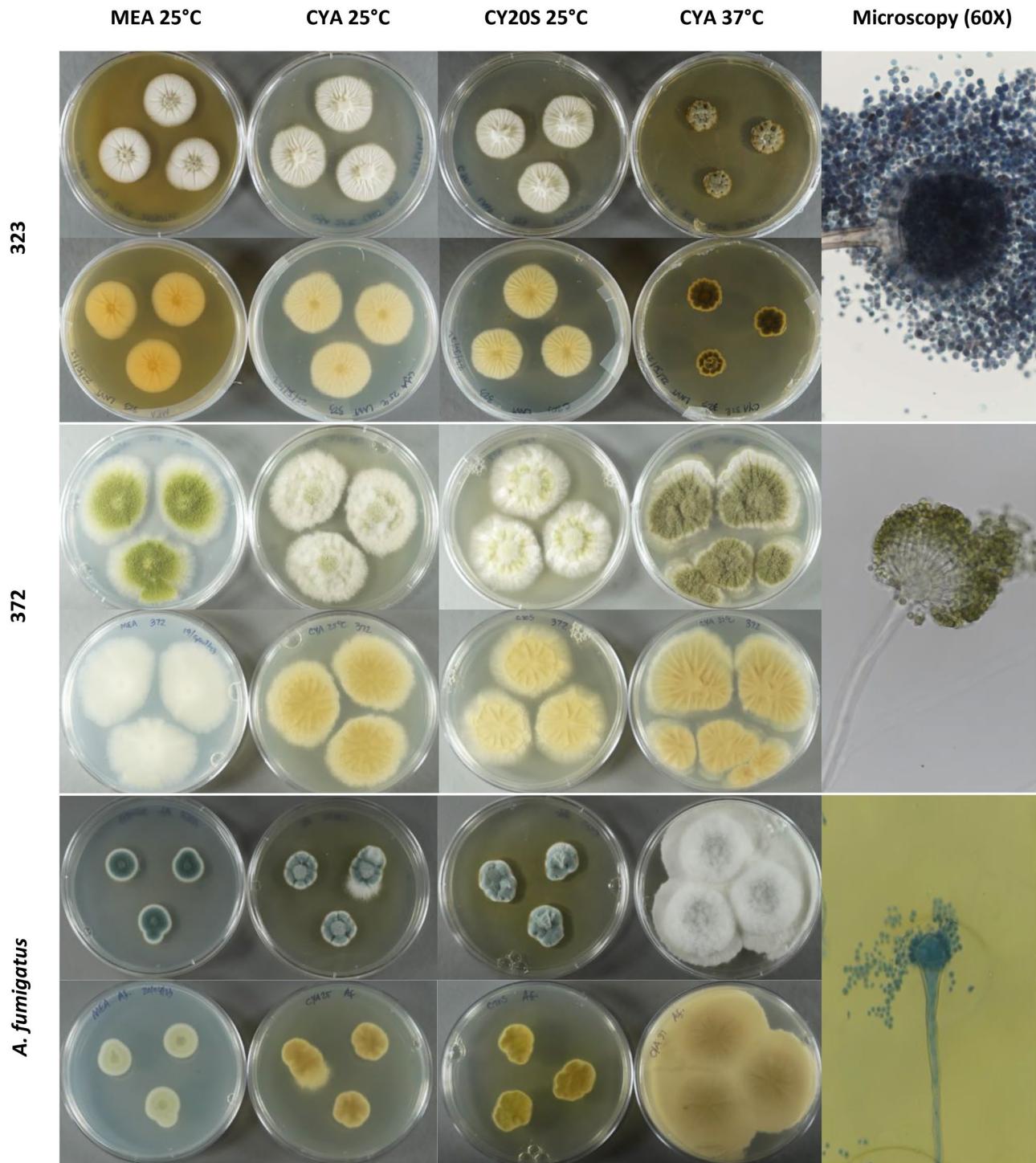
Fig. 1. (continued)

CaM, respectively. While these are generally acceptable for fungal phylogenetics, the limited variability in some regions may contribute to lower support values in specific branches. The study's sample size was limited to 28 isolates from a specific geographic area (San Juan, Puerto Rico), potentially limiting the generalizability of findings to broader environments affected by flooding, such as those that are not hot and humid. Additionally, exclusively relying on morphological keys can lead to unsuccessful identifications, especially when dealing with newly described *Aspergillus* species. The need to revise these keys is indicated as more species are being discovered. We also acknowledge that we did not quantify species-specific airborne concentrations (CFU/ m^3) for each *Aspergillus* isolate. Air sampling produced CFU/m^3 at the genus level during culture, whereas species identification was performed later on representative isolates; because several taxa are phenotypically indistinguishable by routine macro-/micromorphology, colony counts cannot be retrospectively attributed to

Isolate code	Identity of isolate	Identity (%) to GenBank sequences and E-value					
	Molecular approach	ITS2	E-value	*benA*	E-value	*caM*	E-value
19	A. versicolor	100	7e−163	100	0.0	99.80	0.0
20	A. steynii	99.68	1e−159	100	0.0	100	0.0
27	–	–	–	–		–	
33	A. niger	100	5e−164	100	0.0	100	0.0
50	A. arenarioides	100	1e−159	100	0.0	100	0.0
73	–	–	–	–		–	
75	A. chevalieri	100	3e−161	100	0.0	100	0.0
77	A. petersonii	100	1e−159	100	0.0	99.64	0.0
111	A. subalbidus	100	7e−163	100	0.0	100	0.0
137	A. penicillioides	100	2e−168	99.78	0.0	100	0.0
139	A. sydowii	100	4e−160	100	0.0	99.60	0.0
143	A. hordei	100	2e−168	99.77	0.0	100	0.0
150	A. versicolor	100	3e−161	100	0.0	99.80	0.0
155	A. flavus	100	1e−160	100	0.0	100	0.0
159	A. ochraceopetaliformis	100	1e−159	100	0.0	100	0.0
173	A. gracilis	100	1e−165	100	0.0	100	0.0
215	A. westerdijkiae	100	1e−165	100	0.0	100	0.0
226	A. chevalieri	100	1e−160	100	0.0	100	0.0
229	A. tamarii	100	7e−163	100	0.0	100	0.0
232	A. hordei	100	7e−168	99.36	0.0	100	0.0
273	–	–	–	–		–	
276	–	–	–	–		–	
277	–	–	–	–		–	
293	A. flocculosus	99.38	1e−160	99.82	0.0	100	0.0
299	–	–	–	–		–	
321	A. pseudoglaucus	100	8e−157	99.75	0.0	100	0.0
323	A. brunneoviolaceus	100	2e−153	98.38	0.0	100	0.0
372	A. oryzae	100	6e−148	100	0.0	100	0.0
A. fumigatus (DNA extraction control)	A. fumigatus	100	2e−163	100	0.0	99.82	0.0
A. brasiliensis (PCR control)	A. brasiliensis	100	3e−161	100	0.0	100	0.0

Table 3. Molecular-based identification of 29 *Aspergillus* isolates. The percentage of similarity and E-value to reference sequences of GenBank is shown per gene and *Aspergillus* isolate. "–" denotes that no clear PCR products were obtained using primers from Table 4.

species. To provide quantitative context, we included per-site total *Aspergillus* spp. CFU/m^3 and the proportion of *Aspergillus* within total filamentous fungi for the room/areas from which these isolates were recovered (Supplementary Table S1). These are genus-level values for context and should not be interpreted as species-specific concentrations. Future post-disaster studies requiring species-resolved exposure estimates should pair air sampling with parallel molecular quantification at the time of collection (e.g., targeted qPCR). Finally, we did not perform antifungal susceptibility testing; future work should evaluate resistance patterns of environmental *Aspergillus* isolates in the Caribbean, given growing global concern about azole resistance[62].

In conclusion, this study provides essential insights into the variety of *Aspergillus* species in water-impacted homes in a hot and humid environment. It stresses the need for a combination of molecular and morphological techniques for robust fungal identification. Our results on species like *A. versicolor*, *A. flavus*, and *A. sydowii* point to serious implications for indoor air quality and public health in the disaster zone, in favor of extensive identification strategies to inform effective health measures.

Methods
Characterization of *Aspergillus* isolates
Collection of Aspergillus isolates. Approximately 400 *Aspergillus* isolates were cryopreserved in 2.0 mL tubes containing 1 mL of culture media (MEA or G25N) and stored at -80 °C until further use. To lower the quantity of *Aspergillus* isolates to study, we selected *Aspergillus* isolates from sampled homes that —after aggregating across all sampled rooms/areas within the home—met a priori screening criteria of > 1000 CFU/m^3 total *Aspergillus* spp. or > 50% *Aspergillus* spp. of total filamentous fungi. These thresholds were used to account for higher-burden homes and are not health-based exposure limits, as major guidelines do not endorse quantitative cutoff values for indoor mold contamination[57]. Supplementary Table S1 reports the per-site (room/area) *Aspergillus* spp. concentration (CFU/m^3) and percentage for the specific location from which each isolate was recovered, home-level aggregates were used only for selection. Approximately 210 *Aspergillus* isolates recovered from the sampled
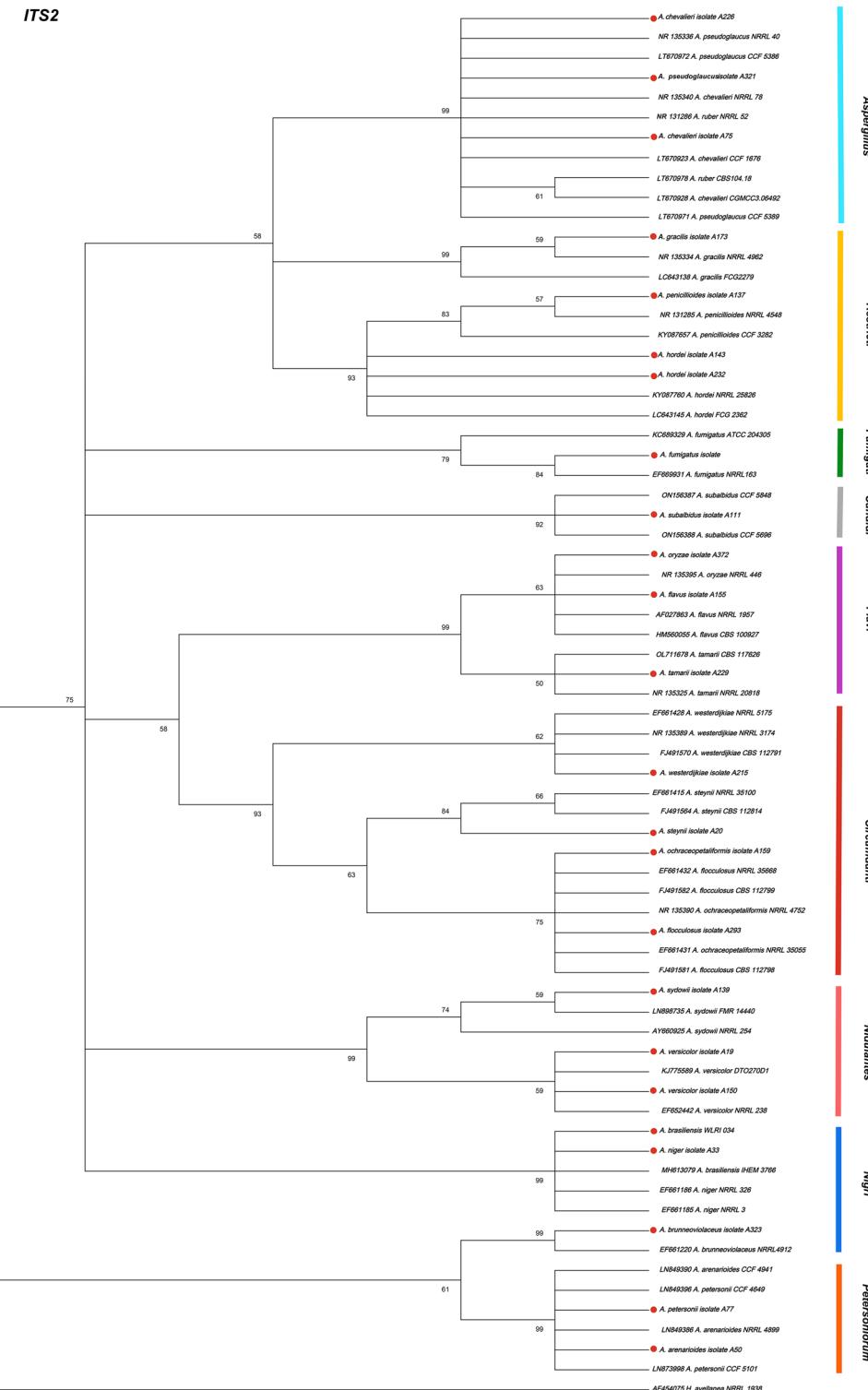
**Fig. 2**. Phylogenetic tree of partial ITS2 gene sequences by maximum likelihood. Sequences from this study are highlighted with a red diamond shape. *Hamigera avellanea* was used as an outgroup. The isolate Section classification is highlighted in color.

homes met these criteria. We randomly selected 28 *Aspergillus* isolates from this list for further testing to balance feasibility and resource constraints, ensuring a manageable sample size for in-depth species identification. These *Aspergillus* isolates were grown on G25N media for up to 14 days at 25 ± 2 °C to generate sufficient growth to identify them at the species level and to perform experiments evaluating their pro-inflammatory potential.
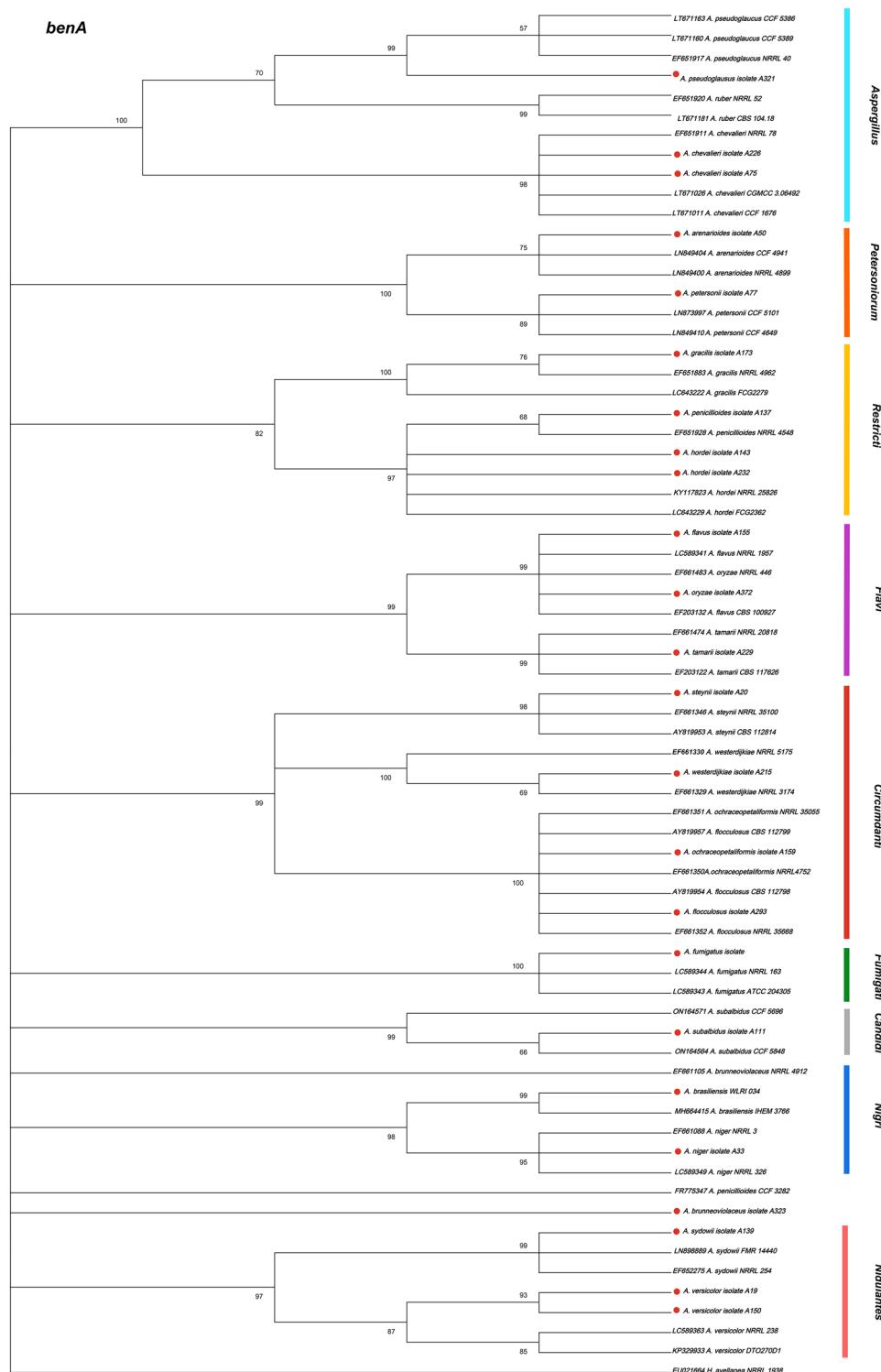
Fig. 3. Phylogenetic tree of partial *benA* gene sequences by maximum likelihood. Note: Sequences from this study are highlighted with a red diamond shape. *Hamigera avellanea* was used as an outgroup.

Macromorphological characterization of Aspergillus isolates
To identify the selected 28 *Aspergillus* isolates to species level through culture techniques, we followed Maren A. Klich's morphologically based system[14]. Colonies were grown on four media to describe the fungal isolates macromorphologically. The media used were the following: Czapek Yeast Agar (CYA, K_2HPO_4 0.5 g, Czapek concentrate 5.0 mL, yeast extract 2.5 g, sucrose 30.0 g, agar 7.5 g, distilled water 500 mL), Czapek Yeast Agar with 20% sucrose (CY20S, K_2HPO_4 0.5 g, Czapek concentrate 5.0 ml, yeast extract 2.5 g, sucrose 100.0 g, agar 7.5 g, distilled water 500 mL) and commercial Malt Extract Agar (MEA, Hardy Diagnostics). Twenty-five mL of
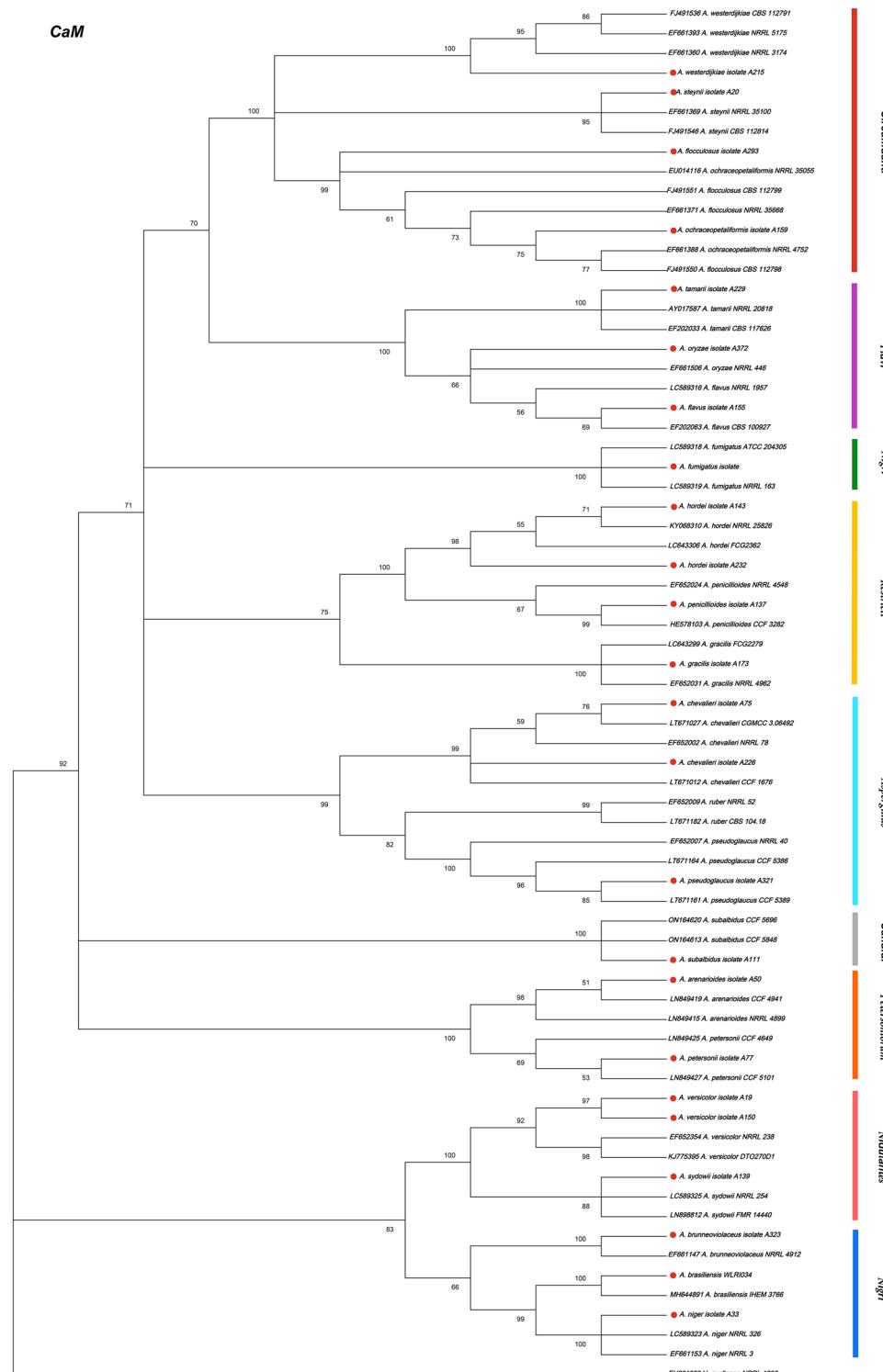
Fig. 4. Phylogenetic tree of partial *caM* gene sequences by maximum likelihood. Sequences from this study are highlighted with a red diamond shape. *Hamigera avellanea* was used as an outgroup. The isolate Section classification is highlighted in color.

sterilized media were poured into standard (100 mm) Petri dishes. Four plates were used for each culture: two of CYA and one of CY20S and MEA. To prevent stray colonies on the plates, we prepared spore suspensions using a medium consisting of 0.2% agar and 0.05 Tween 80. Briefly, we pipetted 1 mL aliquots of the sterilized medium into small 2.5 mL cryovials. We mixed conidia from 7 to 14-day-old growth into the medium. Then, we placed 2 µL aliquots at three equidistant points from the center of the plate. We incubated each plate for seven days, with one CYA plate at 37 °C and the remaining at 25 °C. Incubation at 25 °C represents a typical indoor

Locus	Primer	Direction	Oligonucleotide sequence (5′–3′)	Length (bp)
Internal transcribed spacer 2 (*ITS2*)	ITS9	Forward	GAA CGC AGC RAA IIG YGA	240–460
	ITS4	Reverse	TCC TCC GCT TAT TGA TAT GC	
Beta-tubulin (*benA*)	Bt2a	Forward	GGT AAC CAA ATC GGT GCT GCT TTC	432–560
	Bt2b	Reverse	ACC CTC AGT GTA GTG ACC CTT GGC	
Calmodulin (*CaM*)	CMD5	Forward	CCG AGT ACA AGG ARG CCT TC	580
	CMD6	Reverse	CCG ATR GAC GTC ATR ACG TGG	

Table 4. Primers used for the amplification of specific genes in the *Aspergillus* isolates.

room temperature, whether incubation at 37 °C is done to simulate human body temperature. After the 7-day incubation, we collected data on conidial color, colony diameter, mycelial color, exudate presence, reverse color, soluble pigment, sclerotia, and cleistothecia.

Micromorphological characterization of Aspergillus isolates
We used the microculture technique to describe the micromorphological features of the selected *Aspergillus* isolates. Briefly, we inoculated two parallel lines of the *Aspergillus* conidia from a 7 to 14-day-old colony in a 60 mm Petri Dish with G25N media using an inoculating needle. Then, we inserted a sterile coverslip at a 45-degree angle in each inoculated line. We incubated the microcultures at 25 ± 2 °C for 7 days. After incubating, we gently collected the coverslips using stained (with lactophenol cotton blue) and unstained slide preparations. We used the NIKON 80i microscope to observe and collect micromorphology features such as seriation type (predominantly uniseriate or biseriate), vesicle shape, conidia characteristics (shape, size, and surface texture), stipe (length, color, and surface texture) and ascospores (color, size, ornamentation, and surface texture).

Molecular identification of Aspergillus isolates
We extracted fungal genomic DNA using the standard protocol of Qiagen DNeasy PowerSoil Pro Kit (QIAGEN LLC, Germantown Road, Maryland, USA). For sample preparation, we grew each fungal isolate on G25N and incubated them at 25 ± 2 °C for 7 to 14 days. Then, we transferred fungal growth from the surface of the plate using a sterile swab or a sterile scalpel blade to the bead tube of the DNAeasy PowerSoil Pro Kit and followed the manufacturer's instructions. We quantified the genomic DNA using the Qubit® dsDNA HS (High Sensitivity) Assay at room temperature (Waltham, Massachusetts, US) and stored at − 20 °C until genomic DNA amplification and sequencing.

PCR amplification of target genes
For PCR amplification, we used primers specific for Internal Transcribed Spacer Region 2 (ITS2), beta-tubulin gene (*benA*), and calmodulin gene (*CaM*) (Table 4). We performed PCR amplification of the extracted DNA in a 100 μL reaction mixture as follows: 5 μL gDNA template, 50 μL Qiagen HotStarTaq Master Mix (QIAGEN LLC, Germantown Road, Maryland, USA), 5 μL of each forward and reverse primers, and 35 μL RNase free water. We included a non-template negative and a positive control (genomic DNA from *Aspergillus brasiliensis*, ATCC 16404D-2) in each amplification reaction. We programmed the thermocycler to the following PCR conditions: HotStarTaq DNA Polymerase activation incubation step at 95 °C for 15 min, 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. After complete amplification, we analyzed the PCR products for gel electrophoresis using 1.5% agarose gel (1.5 g of agarose in 100 ml of TAE 1 × buffer) with ethidium bromide as the staining agent.

Sequencing and phylogenetic analysis
We purified the PCR products using the Qiagen QIAquick PCR Purification Kit (QIAGEN LLC, Germantown Road, Maryland, USA), according to the manufacturer's instructions, and sequenced with the primers used for amplification. Sequencing was outsourced using the Big Dye X Terminator Sequencing Kit 3.1 and the ABI 3500 DNA Sequencer (Applied Biosystems) at the Molecular Biology Core Facility from the RCMI Program at the University of Puerto Rico—Medical Sciences Campus. We verified and cleaned the sequences using FinchTV (Geospiza, Inc.) Version 1.5.0 chromatogram viewer software. We assigned species names to the *Aspergillus* isolates after comparing the contigs (created from forward and reverse complement sequences) with representative sequences available in NCBI (National Center for Biotechnology Information). For phylogenetic analysis, we aligned sequences for each gene region (ITS2, beta-tubulin [*benA*], and calmodulin [*CaM*]) using the ClustalW algorithm with default parameters in MEGA version 12[18]. Multiple sequence alignments were visually inspected and trimmed using the partial deletion method with a 95% site coverage cutoff to exclude positions with significant gaps or missing data. Evolutionary relationships were inferred using the Maximum Likelihood method based on the Tamura-Nei model. The best tree topology was selected based on log-likelihood scores from a heuristic search that compared Neighbor-Joining and Maximum Parsimony starting trees. Robustness of the phylogenetic trees was assessed with 1000 bootstrap replicates. Branches with < 50% support were collapsed. Reference sequences for each gene were downloaded from GenBank and are listed in Supplementary Table S3. *Hamigera avellanea* (family *Aspergillaceae*) was used as the outgroup in all phylogenetic trees.

Data availability

The data supporting this study's findings, including datasets generated and analyzed during the research, are available upon request. Due to privacy considerations regarding sample locations and specifics, data access is restricted but can be granted to the corresponding author upon reasonable request. Any shared data will include the minimal dataset necessary to interpret, replicate, and build upon the findings reported in this article.

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Author contributions

L.N.V.T. contributed substantially to the study's conception and design, performed the primary experiments, acquired, analyzed, and interpreted data, created tables and figures, and drafted the manuscript. B.B.R. supported acquiring and analyzing the culture-based experimental approach and contributed to manuscript revisions. F.G.V. assisted in the design of molecular experiments and contributed to manuscript revisions. F.E.R.M., J.P.M., K.K., and H.C. contributed to the review and substantive revisions of the manuscript. Each author has approved the submitted version with their contributions. All authors agree to be personally accountable for their contributions and to address any questions related to the accuracy or integrity of the study, ensuring any concerns are investigated and appropriately documented in the literature.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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