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Genetic diversity and antifungal susceptibility patterns of *Aspergillus nidulans* complex obtained from clinical and environmental sources

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Summary

The molecular epidemiology and antifungal susceptibility of *Aspergillus nidulans* species complex has not been well studied. To evaluate the genetic diversity and antifungal susceptibility patterns of clinical and environmental isolates of *A. nidulans* complex. Sixty clinical and environmental isolates of *Aspergillus* section *Nidulantes* were collected from five countries (Iran, The Netherlands, Spain, Portugal and Greece). The species were molecularly identified by sequencing of β-tubulin gene. The genetic diversity of *A nidulans* complex isolates (n = 54) was determined with a microsatellite genotyping assay. Antifungal susceptibility profile was determined using EUCAST method. The isolates were classified as *A nidulans* (46.7%), *A spinulosporus* (26.6%), *A quadrilineatus* (10%), *A pachycristatus* (3.3%), *A rugulosus* (3.3%), *A unguis* (5%), *A creber*, (1.7%), *A olivicola* (1.7%) and *A sydowii* (1.7%). Thirty-four sequence types (STs) were identified among the 54 *A nidulans* complex isolates. A high level of genetic diversity was found among *A nidulans* sensu stricto strains but low diversity was found among *A spinulosporus* strains. Amphotericin B showed high MICs

to all species. The most active azole was posaconazole (GM = 0.64 mg/L), while itraconazole showed the highest MICs among azoles (GM = 2.95 mg/L). *A spinulosporus* showed higher MICs than *A nidulans* sensu stricto for all antifungals except for micafungin and anidulafungin. Interspecies variations may result in differences in antifungal susceptibility patterns and challenge antifungal therapy in infections caused by *A nidulans*. Differences in the distribution of STs or persistence of multiple STs might be related to the sources of isolation and niche specialisation.

KEYWORDS

amphotericin B, antifungal susceptibility, *Aspergillus nidulans*, *Aspergillus spinulosporus*, cryptic species, genetic diversity, *β-tubulin*

1 | INTRODUCTION

Nosocomial aspergillosis has emerged an increasing concern in healthcare settings especially at high-risk patients and remains as one of the most common leading causes of global mortality over recent decades worldwide.[1] Although *Aspergillus fumigatus* is the most common aetiologic agent of aspergillosis, non-*fumigatus Aspergillus* species such as *Aspergillus flavus* have been reported predominantly in tropical and subtropical countries.[2] Additionally, within recent years, other *Aspergillus* species including *Aspergillus niger* and *Aspergillus nidulans* have also been encountered as emerging species causing infections.[3] The spread and persistence of rare and emerging species can be associated with the extensive use of azoles and amphotericin B as antifungal therapy or prophylaxis.[4,5] Besides, the overuse and misuse of the available antifungals may lead to antifungal resistance.[6] Based on a newly proposed classification,[2] the genus *Aspergillus* contains more than 250 species, which are subdivided into 22 distinct sections. Of these, at least 13 sections, including *Candidi*, *Circumdati*, *Flavipides*, *Fumigati*, *Nidulantes*, *Nigri*, *Ornati*, *Restricti*, *Tanneri*, *Terrei*, *Usti*, *Versicolores* and *Warcupi*, contain clinically relevant species. Section *Nidulantes* comprises a diverse group of species, of which *A nidulans* complex has currently emerged as a serious threat to immunocompromised patients, especially to those with chronic granulomatous disease (CGD).[7] The use of amphotericin B as an empirical and first-line treatments and intrinsically lower susceptibility of *A nidulans* to amphotericin B appear to be the main characteristics contributing to the increasing incidence of infection by the species.[5,8] The genetic diversity and its association with antifungal susceptibility within *A nidulans* species complex have not been described to date.

Simple sequence repeats (SSRs) can be used as an effective molecular marker for genetic, taxonomic, diversity, clonality, disease outbreaks and epidemiologic studies.[9,10] A growing body of evidence has indicated that multiple loci variable tandem repeat analysis (MLVA) can target the number of tandem repeated sequences at different genomic loci to detect the variations between isolates of filamentous fungi, especially for *A fumigatus and A flavus*.[11-13]

We therefore conducted, for the first time to our knowledge, an epidemiological study on *A nidulans* complex isolates obtained from diverse geographical regions and at different time points. All isolates were subjected to molecular phylogenetic analysis using *β*-tubulin region, and their antifungal susceptibility profile was determined. Subsequently, we developed and tested an MLVA subtyping technique to investigate the population diversity of *A nidulans* complex species in a wide geographical area.

2 | MATERIAL AND METHODS

2.1 | Strains

Sixty isolates within the *Nidulantes* section were sequentially collected over a 9-year period (2010-2018) from clinical and environmental sources from five different countries (Table 1). Of these, 47 strains were isolated from clinical samples (respiratory, spinal mass, vertebral bone, nail, skin, ear exudate and abscess aspirate): 26 from the Netherlands, nine from Spain, two from Portugal, five from Greece and five from Iran. Environmental strains came from the air of hospital wards, soil, tree, sewage of Iran (n = 10) and Portugal (n = 3). All strains were delivered to the Mycology Reference Laboratory of the Spanish National Center for Microbiology, Instituto de Salud Carlos III (ISCIII), Spain. The Ethic Committee of Mazandaran University of Medical Sciences, Iran, approved the study (IR.MAZUMS.REC.95.2819.)

2.2 | Molecular identification

The isolates were subjected to molecular analysis as follows. Genomic DNA was extracted from all isolates that were previously subcultured in glucose-yeast extract-peptone liquid medium (0.3% yeast extract, 1% peptone; Difco, Soria Melguizo) with 2% glucose (Sigma-Aldrich Química) for 24 to 48 hours at 30°C, following a method previously described.[14,15] A portion of β-tubulin gene was amplified by PCR using primers previously described.[16] PCR mixtures contained 0.5 μmol/L of each primer, 0.2 mmol/L of each deoxynucleoside triphosphate (Roche), 5 μL of PCR 10× buffer containing $MgCl_2$ (Applied Biosystems), 2.5 U *Taq* DNA polymerase

TABLE 1 Characterisation and molecular identification of the *A nidulans* isolates used in this study

ID	Strain no.	Geographic origin	Date of sampling	Source	GenBank accession number
					BenA
1	2225	Iran	2012-11-21	Spinal mass in CGD patient	MK749983
2	2402	Iran	2013-03-18	Vertebral bone tissue in CGD patient	MK749954
3	1528	Iran	2017	Sputum	MK749943
4	29en	Iran	2012	Tree	MK749963
5	33en	Iran	2012	Soil	MK749964
6	70en	Iran	2012	Sewage	MK749965
7	W-SH 13-1	Iran	2014-11-18	The wall surface of the haematology ward	MK749994
8	W-SH 11-1	Iran	2015-03-02	The wall surface of the haematology ward	MK749993
9	W-M 7-9	Iran	2014-09-14	The wall surface of the haematology ward	MK749990
10	W-M 7-11	Iran	2015-01-05	The wall surface of the haematology ward	MK749992
11	17A	Iran	2016-11-01	Sputum in an asthmatic patient	MK749944
12	IR9	Iran	2016	Air	MK749967
13	W-M-13-2	Iran	2014-10-01	The wall surface of the haematology ward	MK749991
14	W-SH 9-2	Iran	2014-08-18	The wall surface of the haematology ward	MK749995
15	IR14	Iran	2015	Ear exudate	MK749966
16	CM8153	Spain	2015-02-11	Sputum HIV patient	MK749974
17	CM6956	Spain	2011-05-01	Sputum	MK749971
18	CM8425	Spain	2015-03-11	Sputum COPD patient	MK749939
19	CM4877	Spain	2007	Abscess aspirate	MK749968
20	CM6685	Spain	2010-10-01	Sputum	MK749969
21	CM8754	Spain	2016-12-21	Sputum	MK749975
22	CM6772	Spain	2010-10-01	Bronchoaspirate	MK749970
23	CM6992	Spain	2012	BAL	MK749972
24	CM7464	Spain	2014	Unknown	MK749973
25	V235-37	The Netherlands	217-11-28	Sputum	MK749938
26	V259-38	The Netherlands	2018-07-27	Sputum	MK749962
27	V208-33	The Netherlands	2016-09-22	Sputum	MK749985
28	V212-39	The Netherlands	2016-11-29	Sputum	MK749948
29	V212-36	The Netherlands	2016-11-30	Sputum	MK749947
30	V213-28	The Netherlands	2016-12-15	Sputum	MK749986
31	V225-49	The Netherlands	2017-06-30	Sputum	MK749987
32	V227-11	The Netherlands	2017-07-14	Sputum	MK749988
33	V226-78	The Netherlands	2017-07-17	Sputum	MK749949
34	V229-39	The Netherlands	2017-08-25	Sputum	MK749951
35	V230-05	The Netherlands	2017-09-05	Sputum	MK749952
36	V237-66	The Netherlands	2017-12-14	Sputum	MK749953
37	V249-15	The Netherlands	2018-03-21	Sputum	MK749957
38	V248-53	The Netherlands	2018-03-09	Sputum	MK749955
39	V249-76	The Netherlands	2018-03-28	Sputum	MK749958
40	V252-12	The Netherlands	2018-05-01	Sputum	MK749959
41	V252-22	The Netherlands	2018-05-04	Sputum	MK749960
42	V257-26	The Netherlands	2018-07-03	Sputum	MK749961
43	V258-68	The Netherlands	2018-07-18	Sputum	MK749989

(Continues)

TABLE 1 (Continued)

ID	Strain no.	Geographic origin	Date of sampling	Source	GenBank accession number
					BenA
44	V254-64	The Netherlands	2018-06-01	Sputum	MK749984
45	V233-35	The Netherlands	2017-10-27	Sputum	MK749937
46	V248-63	The Netherlands	2018-03-14	Sputum	MK749956
47	V232-56	The Netherlands	2017-10-18	Sputum	MK749936
48	V207-28	The Netherlands	2016-08-26	Sputum	MK749945
49	V209-02	The Netherlands	2010-07-16	Sputum	MK749946
50	V229-12	The Netherlands	2017-08-17	Sputum	MK749950
51	INSA5	Portugal	2012-08-22	Undetermined	MK749982
52	INSA11	Portugal	2012-05-18	Undetermined	MK749981
53	HSMA46	Portugal	Sep 2012	ICU ward	MK749978
54	HSMA48	Portugal	Sep 2012	Haematology ward	MK749979
55	HSMA55	Portugal	Dec 2012	BM transplantation ward	MK749980
56	GR109	Greece	2010-12-09	Nail clippings	MK749940
57	GR138	Greece	2011-02-15	Nail clippings	MK749976
58	GR701	Greece	2013-10-29	Skin scrapings	MK755459
59	GR1214	Greece	2018-02-08	BAL	MK749977
60	GR1268	Greece	2018-04-20	Nail clippings	MK749941

(Amplitaq; Applied Biosystems) and 25 ng of DNA in a final volume of 50 μL. The samples were amplified in a GeneAmp PCR System 9700 (Applied Biosystems) using an initial cycle of 2 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 45 seconds at 56°C, 2 minutes at 72°C and one final cycle of 5 minutes at 72°C. Reactions products were analysed in a 0.8% agarose gel. In some cases, amplification products yielded two bands with different size, in which event 40 μL of the amplified product was loaded in a 0.8% agarose gel and allowed to run until the two bands were well separated. Each of them was individually excised from the gel and purified using QIAquick Gel Extraction kitÒ protocols (Qiagen Inc). Otherwise, PCR products were purified using Illustra ExoProStar 1-step (GE Healthcare Lifescience).

PCR purified products were sequenced by Sanger method with an ABI3730XL sequencer (Applied Biosystems). Sequences were analysed using DNAStar Lasergene 12 software (DNASTAR, Inc). Sequences were compared with reference sequences from type strains obtained from GenBank database. Thus, *A rugulosus* strain NRRL 206 (sequence EF652258), *A pachycristatus* strain NBRC 104790 (AB375875), *A nidulans* strain CBS 589.65 (EF652251), *A quadrilineatus* strain NRRL 201 (EF652257), *A unguis* strain CBS 132.55 (EF652257), *A spinulosporus* strain NRRL 2395 (AY573553), *A sydowii* strain CBS 553.65 (EF652274), *A creber* strain NRRL 58592 (JN853980) and *A olivicola* strain CBS 119.37 (AY339996) were included in the sequence comparison. Phylogenetic analysis was conducted with InfoQuest Package v. 4.5. (Biorad), a bootstrap analysis was performed with 2000 simulations (Figure 1). The obtained sequences were deposited in the GenBank under accession numbers MK749936 to MK749995 and MK755459 (Table 1).

2.3 | Development of MLVA scheme

Eight SSRs primer pairs designed by Hosid et al[17] were selected for testing. The 5′ end of each forward primer was labelled with a fluorescent dye, 6-carboxyfluorescein (FAM; Eurogentec). Despite being part of section *Nidulantes*, isolates classified as *A sydowii*, *A creber*, *A olivicola* and *A unguis* were not included in the MLVA analysis because of their higher genetic distance with *A nidulans* (Figure 1). Therefore only *A nidulans* complex isolates (the ones that were morphologically and genetically more related with *A nidulans* sensu stricto) were included in this analysis.

Optimised PCRs were performed for each SSR loci in a 20 μL volume containing 10 ng template DNA, 2 μL PCR 10× buffer, 1.5 mmol/L $MgCl_2$, 2.5 mmol/L dNTPs, 10 mmol/L each of FAM-labelled primer (Applied Biosystems), 10 mmol/L reverse primer and 0.5 U of Taq DNA polymerase. PCR conditions were set as follows: 95°C for 3 minutes; 45 cycles of 94°C for 1 minute, 52°C for 45 seconds, and 72°C for 30 seconds; and 72°C for 7 minutes. Fluorescently labelled amplicons were analysed in a 2% agarose gel.

Amplicons were diluted 1:20, and 1 μL of each was mixed with 9 μL of Hi-Di Formamide (Thermo Fisher Scientific) and 1 μL of GeneScan 500 ROX size standard (Applied Biosystems) for separation by capillary electrophoresis on an ABI genetic analyser (Applied Biosystems). Fragment analysis was performed using PeakScanner software (Applied Biosystems).

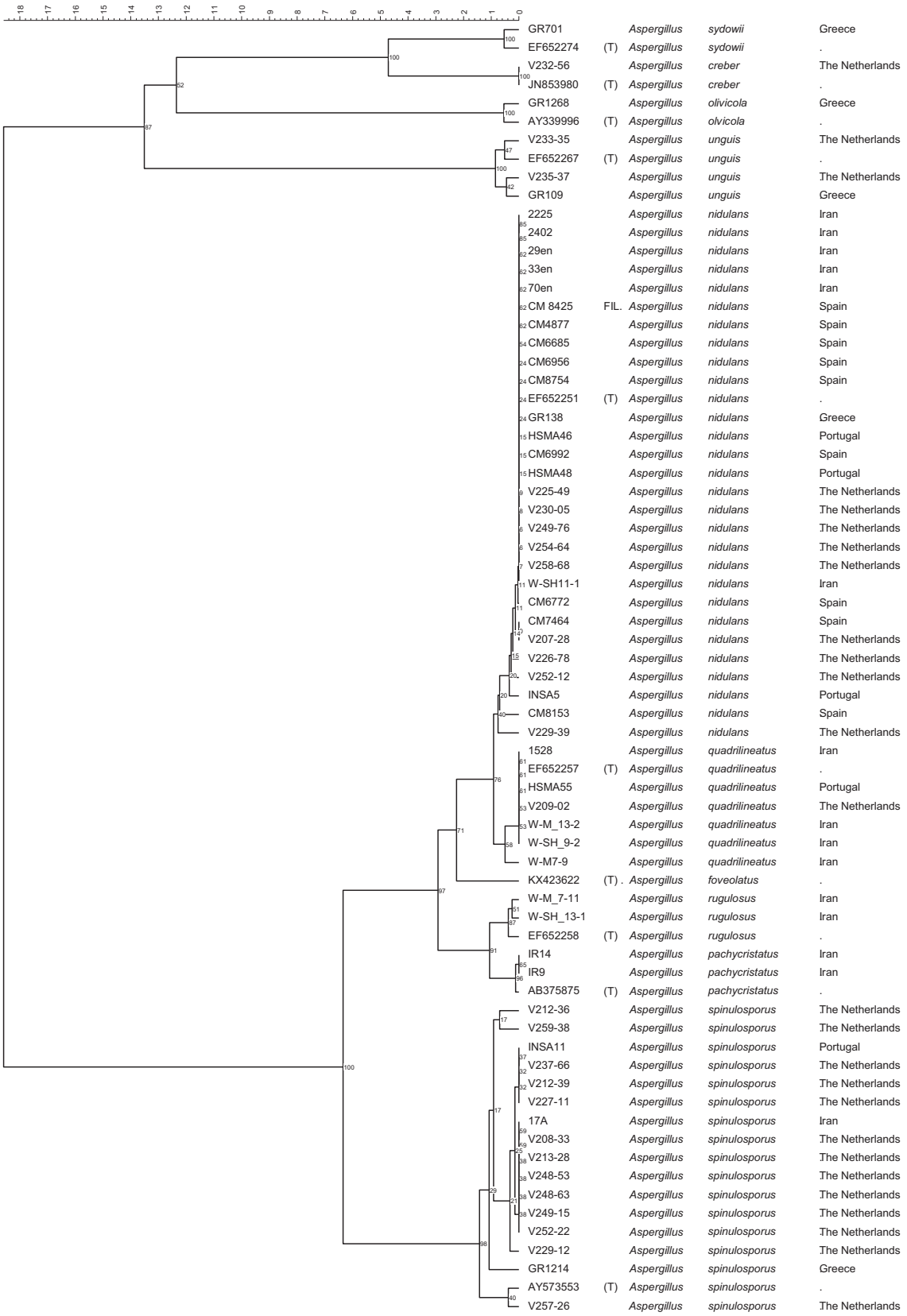

FIGURE 1 BenA phylogenetic tree. The tree was constructed by the UPGMA method. Bootstrap values of 2000 replications are estimated on branch-points

TABLE 2 Features of the SSRs loci composing the newly developed MLVA scheme

Locus name	Localisation	Tandem repeat Sequence	Motif size (bp)	Forward primer (5′-3′)	Reverse primer (5′-3′)
NC8L1	Chr VIII	$(GCA)_{10}$	127-154	FAM-TCAGAGGATCCAGGACGACTAG	GACCTGTGTCACCTACGACTGC
NC8L2	Chr VIII	$(CTT)_{7}$	162-188	FAM-CGTTGGCTGTGGTTACCGAC	TGGACTGATTGCCGGGTTAATC
NC2L1	Chr II	$(GCA)_{25}$	216-373	FAM-CAGGAGTTGGCGACATCGTCTG	GTTTCGGTCCCTGGTTTCTGTGTC
NC8L4	Chr VIII	$(CAG)_{12}$	132-199	FAM-ACTCCGCAACAGTTCGCTCAG	TCTGAGCCTGGTATGTCTGGG

2.4 | Statistical analysis

Statistical analyses were performed with SPSS v.19.0. The chi-square test or Fisher's exact test were used for the assessment of association between STs, isolation source and the origin of isolates.

2.5 | In vitro antifungal susceptibility testing using EUCAST

The minimum inhibitory concentrations (MICs) of itraconazole (Sigma-Aldrich Química), posaconazole (Schering-Plough Research Institute), voriconazole (Pfizer SA), amphotericin B (Sigma-Aldrich Química), terbinafine (Novartis) and the minimum effective concentrations (MECs) of anidulafungin (Pfizer SA), caspofungin (Merck & Co., Inc) and micafungin (Astellas Pharma, Inc) were evaluated using the EUCAST guidelines for broth microdilution testing of filamentous fungi.[18] The final concentrations tested ranged from 0.015 to 8.0 mg/L for itraconazole, voriconazole and posaconazole, from 0.03 to 16 mg/L for amphotericin B, terbinafine and caspofungin, 0.004-2.00 mg/L for micafungin, and 0.007-4.00 mg/L for the anidulafungin. After incubation of microplates at 35°C, MIC and MEC readings were made at 24 and 48 hours by the use of a mirror.[19] Geometric mean (GM) and $MIC/MEC_{50}/_{90}$ (MIC/MEC inhibiting the growth of 50% or 90% of the strains tested) values were calculated. *A flavus* (ATCC 2004304) and *A fumigatus* (ATCC 2004305) were used as quality control strains. For calculation purposes, the MIC/MEC values that exceeded the maximum concentration tested were transformed to the next twofold higher dilution (ie if MIC/MEC was >8 mg/L it was expressed as 16 mg/L).

3 | RESULTS

3.1 | Identification of strains

Out of 60 collected isolates, the strains were classified as *A nidulans* (28, 46.7%), *A spinulosporus* (16, 26.6%), *A quadrilineatus* (6, 10%), *A unguis* (3, 5%), *A pachycristatus* (2, 3.3%), *A rugulosus* (2, 3.3%), *A creber*, (1, 1.7%), *A olivicola* (1, 1.7%) and *A sydowii* (1, 1.7%) all belong to section *Nidulantes*, based on β-tubulin sequencing results (Figure 1). In five out of 16 *A spinulosporus* isolates and all *A rugulosus* isolates analysed, two main bands were present after PCR amplification and were purified and sequenced directly from the gel. After sequencing and performing a BLAST search, we concluded that both bands were derived from β-tubulin paralogues, one band was represented by *benA* (the targeted gene) and the other was represented by *tubC*. In these cases, *benA* was included in the phylogenetic tree to identify the isolate.

3.2 | Sequence types (STs)

Fifty-four isolates belonging to the *A nidulans* complex were genotyped by PCR amplification of a set of eight SSR markers, as listed in Table 2, in order to study DNA sequence variation patterns. All loci could be amplified for every isolate; however, the allelic variability of four of the tested loci (NC6l1, NC8L5, NC6L3, NC1L1) was limited or null for all isolates and was, thus, excluded from the final selection of markers. The remaining four loci (NC8L1, NC8L2, NC8L4, NC2L1) were found to be polymorphic. Based on the combination of all four loci, 35 sequence types (STs) were identified among 54 isolates (Table 3). Each species had a unique ST that was not shared with other species of the complex. Overall, 50% (27/54) of the strains consisted of individual genotypes with only one representative isolasted from clinical or environmental sources.

The remaining 27 isolates had genotypes that were shared with other isolates in the study and were found in both clinical and environmental isolates. We found 23 sequence types in 28 isolates of *A nidulans* sensu stricto (ST1, ST3-5, ST7, ST12-18, ST21-28, ST30, ST32 and ST34), five sequence types in 16 *A spinulosporus* isolates (ST9, ST19, ST20, ST31 and ST35), five sequence types in six *A quadrilineatus* isolates (ST2, ST8, ST11, ST29 and ST33), and *A pachycristatus* and *A rugulosus* (each species with two isolates) with a single sequence type: ST10 and ST6, respectively. Thirteen strains of *A spinulosporus* were originated from the Netherlands and represented three sequence types, of which ST9 and ST20 were the most frequently found. The remaining three isolates of *A spinulosporus* were originated from Iran, Portugal and Greece and belonged to ST9, ST31 and ST35, respectively.

A total of 38 (70.4%) different microsatellite genotypes belonging to the following 24 (68.6%) different subtypes ST1, ST2, ST9, ST12, ST13, ST15, ST15-31 and ST34 and ST35 were present only in clinical samples. From these, respiratory samples were the most frequent sources (32 isolates or 84.3%), of which 29 were from respiratory secretions including sputum (90.6%), two from BAL (6.3%), and one from bronchoaspirate fluid (3.1%). However, isolates collected from other biological sample were also analysed:

TABLE 3 Sequence typing of 54 strains of *A nidulans* complex isolated from different clinical and environmental specimens obtained from Asian and European countries

ID	Strain no.	Species	NC8L1 (bp)	NC8L2 (bp)	NC8L4 (bp)	NC2L1 (bp)	Sequence type
1	2225	*A nidulans*	145	178	152	300	ST1
2	2402	*A nidulans*	145	178	152	301	ST1
3	1528	*A quadrilineatus*	132	184	176	364	ST2
4	29en	*A nidulans*	151	175	176	316	ST3
5	33en	*A nidulans*	160	178	190	301	ST4
6	70en	*A nidulans*	151	175	179	316	ST5
7	W-SH 13-1	*A rugulosus*	136	181	154	332	ST6
8	W-SH 11-1	*A nidulans*	148	178	167	301	ST7
9	W-M 7-9	*A quadrilineatus*	145	184	170	364	ST8
10	W-M 7-11	*A rugulosus*	136	181	153	332	ST6
11	17A	*A spinulosporus*	138	184	150	270	ST9
12	IR9	*A pachycristatus*	136	181	153	226	ST10
13	W-M-13-2	*A quadrilineatus*	145	184	179	335	ST11
14	W-SH 9-2	*A quadrilineatus*	145	184	179	335	ST11
15	IR14	*A pachycristatus*	136	181	153	226	ST10
16	CM8153	*A nidulans*	148	178	176	301	ST12
17	CM6956	*A nidulans*	129	179	173	301	ST13
18	CM8425	*A nidulans*	148	178	176	301	ST12
19	CM4877	*A nidulans*	138	178	173	316	ST14
20	CM6685	*A nidulans*	154	178	152	301	ST15
21	CM8754	*A nidulans*	148	178	176	301	ST12
22	CM6772	*A nidulans*	135	182	160	316	ST16
23	CM6992	*A nidulans*	151	178	152	301	ST17
24	CM7464	*A nidulans*	138	178	176	316	ST18
25	V259-38	*A spinulosporus*	134	184	150	361	ST19
26	V208-33	*A spinulosporus*	134	184	150	270	ST20
27	V212-39	*A spinulosporus*	138	184	150	270	ST9
28	V212-36	*A spinulosporus*	134	184	150	270	ST20
29	V213-28	*A spinulosporus*	138	184	150	270	ST9
30	V225-49	*A nidulans*	148	175	190	316	ST21
31	V227-11	*A spinulosporus*	138	184	150	270	ST9
32	V226-78	*A nidulans*	148	178	196	301	ST22
33	V229-39	*A nidulans*	151	175	176	316	ST3
34	V230-05	*A nidulans*	142	178	190	316	ST23
35	V237-66	*A spinulosporus*	138	184	150	270	ST9
36	V249-15	*A spinulosporus*	134	184	150	270	ST20
37	V248-53	*A spinulosporus*	134	184	150	270	ST20
38	V249-76	*A nidulans*	148	178	193	301	ST24
39	V252-12	*A nidulans*	148	178	190	316	ST25
40	V252-22	*A spinulosporus*	138	184	150	270	ST9
41	V257-26	*A spinulosporus*	134	184	150	270	ST9
42	V258-68	*A nidulans*	145	178	173	316	ST26
43	V254-64	*A nidulans*	145	178	188	316	ST27
44	V248-63	*A spinulosporus*	134	184	150	270	ST20

(Continues)

TABLE 3 (Continued)

ID	Strain no.	Species	NC8L1 (bp)	NC8L2 (bp)	NC8L4 (bp)	NC2L1 (bp)	Sequence type
45	V207-28	*A nidulans*	151	178	187	316	ST28
46	V209-02	*A quadrilineatus*	148	184	176	364	ST29
47	V229-12	*A spinulosporus*	138	184	150	270	ST9
48	INSA5	*A nidulans*	148	151	190	316	ST30
49	INSA11	*A spinulosporus*	155	185	150	270	ST31
50	HSMA46	*A nidulans*	151	178	173	301	ST32
51	HSMA48	*A nidulans*	138	178	173	316	ST14
52	HSMA55	*A quadrilineatus*	151	184	165	364	ST33
53	GR138	*A nidulans*	144	178	187	316	ST34
54	GR1214	*A spinulosporus*	154	170	150	270	ST35

nail scrapings, bone tissue (vertebrae) and spinal mass (one or 2.6% each); in 7.9% of the isolates, the origin of the biological sample was unknown. The majority of the clinical isolates were from the Netherlands (25 isolates or 65.8%), followed by Spain (five isolates, 13.1%), Iran (four isolates or 10.5%), Portugal and Greece (two isolates each or 5.3%). Thirteen genotypes (18.5%),

TABLE 4 Antifungal susceptibility results for *A nidulans* complex strains, as determined by EUCAST broth microdilution method

	MIC (mg/L)					MEC (mg/L)		
Species (No.)	AMB	ICZ	VCZ	PCZ	TRB	CPF	MCF	ANF
A nidulans (28)								
GM	3.90	0.32	0.21	0.16	0.38	0.44	0.05	0.03
MIC_{50}	2.00	0.25	0.25	0.12	0.25	0.50	0.06	0.03
MIC_{90}	32.00	0.50	0.25	0.25	2.00	1.00	0.06	0.06
Range	0.25-32	0.06-16	0.12-1	0.06-4	0.12-32	0.25-8	0.015-0.25	0.015-0.25
A spinulosporus (16)								
GM	6.17	5.42	2.48	1.83	3.08	3.22	0.08	0.05
MIC_{50}	4.00	8.00	2.00	0.50	2.00	4.00	0.12	0.06
MIC_{90}	32.00	16.00	16.00	16.00	32.00	8.00	0.12	0.12
Range	0.5-32	1-16	0.25-16	0.12-16	0.25-32	1-8	0.03-0.12	0.015-0.12
A quadrilineatus (6)								
GM	2.00	1.59	0.71	0.25	0.63	2.00	0.19	0.27
MIC_{50}	2.00	1.00	0.50	0.25	0.25	2.00	0.12	0.25
MIC_{90}	4.00	16.00	16.00	0.50	32.00	4.00	2.00	8.00
Range	0.5-32	1-16	0.25-16	0.12-16	0.25-32	1-8	0.03-2	0.015-8
A pachycristatus (2)								
Strain: IR14	1.00	0.50	0.25	0.12	1.00	0.50	0.06	0.06
Strain: IR9	>16	1.00	>8	0.25	>16	1.00	0.25	0.12
A rugulosus (2)								
Strain: W-M 7-11	2.00	1.00	0.50	0.12	0.50	2.00	0.12	0.12
Strain: W-SH 13-1	>16	8.00	1.00	0.50	>16	0.50	0.06	0.12
A nidulans complex (54)								
GM	4.16	2.95	1.76	0.64	2.71	2.00	0.13	0.13
MIC_{50}	4.00	2.00	1.00	0.25	1.00	2.00	0.12	0.12
MIC_{90}	32.00	16.00	16.00	16.00	32.00	4.00	0.25	0.50
Range	0.25-32	0.5-16	0.25-16	0.12-16	0.25-32	0.5-4	0.06-2	0.06-8

Abbreviations: AMB: amphotericin B, ICZ: itraconazole, VCZ: voriconazole, PCZ: posaconazole, TRB: terbinafine, CPF: caspofungin, MCF: micafungin (MCF), ANF: anidulafungin, GM: geometric mean. MIC_{50}: MIC inhibiting 50% of the isolates tested, MIC_{90}: MIC inhibiting 90% of the isolates tested.

represented by eight (22.8%) different subtypes ST4-ST8, ST11, ST32 and ST33, were present only in environmental samples. In addition, three sequence types (8.6%) (ST3, ST10 and ST14) were found both in clinical and environmental isolates.

3.3 | Antifungal susceptibility testing

The MIC/$MEC_{50,}$ MIC/MEC_{90}, GM and MIC/MEC ranges of all the antifungals tested against species of *A nidulans* complex with more than five isolates are shown in Table 4. For *A pachycristatus* and *A rugulosus*, the MIC/MECs per strain are shown. Amphotericin B showed high MICs values to all species tested (GM = 4.16 mg/L). The most active azole was posaconazole with GM = 0.64 mg/L, while itraconazole showed the extreme MICs among azoles (GM = 2.95 mg/L), followed by terbinafine (GM = 2.71 mg/L). Micafungin and anidulafungin showed low MECs (GMs = 0.13 mg/L). Regarding differences between species, GM of MICs for *A spinulosporus* isolates was higher than those of *A nidulans* sensu *stricto* isolates for amphotericin B (GM 6.17 vs 3.9 mg/L). Voriconazole and posaconazole with MIC_{90} values of 0.25 mg/L, and terbinafine and caspofungin with the MIC/MEC_{90} values of 2 and 1 mg/L were higher for *A nidulans* sensu stricto compared to *A spinulosporus* which had MIC/MEC_{90} values of 16 mg/L for voriconazole and posaconazole, and 32 and 8 mg/L for terbinafine and caspofungin.

According to the established breakpoint for itraconazole and *A nidulans* and applying it to all species of the complex, 68.3% of the clinical isolates and 84.6% of the environmental isolates were susceptible (MIC ≤ 1). Resistance (MIC > 2) was observed in 26.8% of clinical strains, of which 90.9% were *A spinulosporus* and 9.1% were *A nidulans* sensu stricto. Of 13 environmental isolates, one *A quadrilineatus* and one *A rugulosus* were resistant. Intermediate profile (MIC ≤ 1-MIC > 2) was observed in two clinical isolates (4.9%) of *A spinulosporus*. For amphotericin B, the MIC_{90} value was 32 mg/L being the same among the isolates of *A nidulans* sensu stricto and *A spinulosporus* and with no differences between clinical and environmental isolates.

3.4 | Association within sequence types

We found statistically significant associations between sequence type and isolation source (P = .016, chi-square test). In addition, a significant association (P = .005, Fisher's exact test) was observed between sequence types with the origin of the isolates (clinical or environmental), but no significant association between MIC and isolation sources was found (P > .05, chi-square test; P > .05, Fisher's exact test). The most prevalent sequence types for clinical isolates were ST9 and ST20 accounting for 21% (8/38) and 13.1% (5/38), respectively, of all isolates recovered from sputum.

4 | DISCUSSION

A collection of 60 clinical and environmental isolates belonging to *Aspergillus* section *Nidulantes* identified by classical and molecular methods, obtained from five different countries, was studied. The most frequent species from both clinical and environmental isolates from Iran, the Netherlands, Spain, Portugal, and Greece were *A nidulans* sensu stricto (51.9% isolates), followed by *A spinulosporus* (29.6% isolates), *A quadrilineatus* (11.1% isolates), and *A pachycristatus* and *A rugulosus* (3.7% isolates each). In addition, three *A unguis*, one *A sydowii*, one *A creber* and one *A olivicola*, were also identified among the studied isolates. Despite these later species being part of *Nidulantes* section, we decided not to include them in further analysis for two reasons: because they are less closely related to the *A nidulans* clade (according to Chen et al[20]) and also because of the limited number of strains per species that could be analysed. Therefore, we aimed to provide insight into the significance of geographic distributions, different sources, interspecies variations of *A nidulans* complex and the antifungal activity based on the MIC values, and to analyse the genetic diversity and molecular epidemiology of this complex.

In most isolates of the section, amplification and sequencing of *benA* region was possible, but for some isolates two bands were amplified using the same PCR conditions. After sequencing, we observed that the two regions corresponded to a fragment of *benA* and the other one aligned with *tubC* region. Previous studies have already detected this double amplification of the *tubC* paralogue.[21] Other authors have reported that BT2 primers can amplify a fragment containing two or three introns from *benA* gene and/or another fragment with two introns from the *tubC* paralogue.[22-24] With the co-amplification of both two-intron fragments of β-tubulin *benA* and paralogue *tubC*, the latter product may be misidentified as the *benA* product and cause incongruences between phylogenetic dataset and taxonomic discrepancies, as demonstrated in *Aspergillus* section *Nigri*.[25] In our case, 5 out of 16 *A spinulosporus* isolates (all from the Netherlands) and all *A rugulosus* isolates (from Iran) co-amplification of *tubC* paralogue and *benA* were detected and had to be sequenced after band extraction.

In vitro antifungal susceptibility of the *A nidulans* complex isolates showed that posaconazole, micafungin and anidulafungin had the lowest GMs (<1 mg/L) for *A nidulans* complex, while amphotericin B showed the highest MICs (GM = 4.16 mg/L). Interestingly, MIC/MECs for *A spinulosporus* were higher than those for *A nidulans* sensu stricto for all antifungals tested. Verweij et al[26] reported MICs of 33 strains of *A nidulans* sensu stricto and *A quadrilineatus*. Higher MICs to amphotericin B (MIC 2.5 vs 0.5 mg/L) and lower MECs to caspofungin (0.32 vs 1.83 mg/L) in *A nidulans* sensu stricto isolates compared to *A quadrilineatus* were found.[26] Whilst, terbinafine was the most active antifungal in vitro against both species.[26] In our study, only six isolates of *A quadrilineatus* were tested so clear conclusions cannot be drawn, but this species showed higher MIC/MECs to azoles and caspofungin and lower to amphotericin B than *A nidulans* sensu stricto. Four isolates of *A rugulosus* were tested in a previous study[26] showing low MICs (≤1 mg/L) for all antifungals tested; however, the two isolates we tested in this study had higher MICs, especially one of them that showed MICs > 2 for amphotericin B, itraconazole and terbinafine. We found no data in the literature for *A pachycristatus*, but the

two isolates we tested showed also variable results and, therefore, no clear conclusions can be drawn until more isolates are tested. Also, it has to be taken into account that cryptic species of *A nidulans* might have been misidentified and therefore available in vitro data could represent all species of the complex. More studies are warranted with molecularly identified isolates to have a better idea about the susceptibility profile of these species and also their implication in the clinical setting. The efficacy of amphotericin B, voriconazole and caspofungin has been evaluated in disseminated infections by *A nidulans* compared to other species of *Aspergillus*.[27-29] Of these three antifungals tested, voriconazole and caspofungin were more active and prolonged survival in *A nidulans*[28,29] while amphotericin B provide no significant improvement in survival.[29-32]

Our results indicated that the majority of the isolates were represented by individual sequence types and that those were unique among strains of clinical or environmental origin, except for some sequence types shared by clinical and/or environmental isolates. A significant association was observed between the distribution of sequence types and the MIC/MECs of the different antifungals. The highest level of genetic diversity was found in *A nidulans* sensu stricto isolates represented by 23 sequence types with a high amphotericin B MIC (MIC > 2 mg/L). In addition, *A spinulosporus* isolates with higher MICs to voriconazole, itraconazole, and terbinafine (MIC > 2 mg/L), and posaconazole (MIC > 0.25 mg/L) showed five sequence types. The genetic diversity of sequence types assigned to isolates might be influenced by geographical regions or by ecological factors of the region from where the isolate was obtained. In the current study, a wide spectrum of diverse subtypes was found in each geographical region; however, genotype-by-environment interactions may contribute to increase particular subtypes, as previously demonstrated by other authors.[33] Notably, 13 out of the reported 16 strains of *A spinulosporus* were from the Netherlands and all were isolated from sputum. From those, about half (48.1%) of the isolates were represented by two sequence types (ST9 and ST20), suggesting the subtypes may be adapted to the environment of the country and adding a possible bias to the genotype distribution. Therefore, the observed persistent ST9 and ST20 assigned to *A spinulosporus* isolates recovered from sputum suggested a possible survival overtime or contamination by the same sequence types. More isolates of *A spinulosporus* should be analysed from different origins in order to properly investigate the genetic diversity of this cryptic species.

In this study, we have developed an MLVA scheme, as a potential epidemiological tool. We found genotypic diversity within the species of *A nidulans* complex isolated from both clinical and environmental sources and also from different geographical regions. Interspecies variations may result in differences in antifungal susceptibility patterns and challenge antifungal therapy in infections caused by *A nidulans*. Differences in the distribution of STs or persistence of multiple STs might be related to the sources of isolation and niche specialisation.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

MTH S. S. and A. A. I. have made substantial contributions to conception and design, and given final approval of the version to be published; M. A., R. S., H. Z., S. N. S., H. L. and RV have contributed to acquisition of data; MT and O. R. M. involved in practical activities and drafting the manuscript; J. Y. C. and J. M. were involved in analysis and interpretation of data.

ETHICAL APPROVAL

Ethical approval and patient consensus was not considered necessary due to the study design that based on isolates originated from only the clinical samples obtained during routine laboratory activity. However, the Ethic Committee of Mazandaran University of Medical Sciences, Iran approved the study (IR.MAZUMS.REC.95.2819.)

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