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Original Article

Diversity and Distribution of *Aspergillus fumigatus* and Its Related Species in Izu and Ogasawara Islands, Japan

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ABSTRACT

The taxon *Aspergillus* section *Fumigati* comprises several causative agents of aspergillosis. Here, the distribution of *Aspergillus* sect. *Fumigati* in outdoor environments of Izu and Ogasawara Islands was investigated. Different strains were isolated from soil samples collected from 68 sites on 9 islands (Izu-oshima, Toshima, Shikinejima, Kozushima, Miyakejima, Hachijojima, Mukojima, Chichijima, and Hahajima), including different landscapes, and identified using morphological characteristics and calmodulin (*CaM*) sequences. Seven *Aspergillus* sect. *Fumigati* species were identified. The occurrence frequency of *Aspergillus fumigatus* was higher in forest sites on the islands, except for Ogasawara Islands, whereas that of species other than *A. fumigatus* was higher in bare land and grassland sites on all islands. The occurrence frequency of *A. fumigatus* was more than 50% on islands between Izu-oshima and Toshima, decreased on islands between Shikinejima and Hachijojima, and was zero on Ogasawara Islands. Considering other *Aspergillus* species, *Aspergillus felis* showed high occurrence frequency on islands between Izu-oshima and Shikinejima, *Aspergillus pseudoviridinutans* on islands between Kozushima and Hachijojima, and *Aspergillus udagawae* on Ogasawara Islands. At two study sites (grassland and forest sites), the soil was sampled throughout the year to evaluate whether the occurrence frequency of each fungal species was affected by sampling season. At the grassland site, *A. pseudoviridinutans* was isolated at more than 90% frequency, regardless of the sampling season. *A. fumigatus* occurrence frequency at the forest site ranged from 0% to 60% and greatly varied among sampling seasons. Thus, differences in island location and landscape affected the distribution of *Aspergillu*s sect. *Fumigati*.

Key words : *Aspergillus* section *Fumigati*, biogeography, *CaM*, series *Viridinutantes*, soil

Introduction

Aspergillus section *Fumigati* is a taxon that includes a number of causative agents of fatal aspergillosis in large animals, including humans. This section was classified by Houbraken et al.[1)] into eight series; namely, *Brevipedes*, *Fennelliarum*, *Fumigati*, *Neoglabri*, *Spathulati*, *Thermomutati*, *Unilaterales*, and *Viridinutantes*, with a total of 59 species. Among the species belonging to this section, pathogenicity has been reported for *Aspergillus fumigatus* and *Aspergillus lentulus* of ser. *Fumigati*, *Aspergillus udagawae* of ser. *Viridinutantes*, *Aspergillus hiratsukae* of ser. *Unilaterales*, and *Aspergillus thermomutatus* of ser. *Thermomutati*[2)]. In particular, *A. fumigatus* is the most important species, as it is the causative agent of more than 300,000 cases of aspergillosis annually worldwide[3)]. *A. fumigatus*-related species, which are genetically similar to *A. fumigatus*, belong to another taxon that has recently received clinical attention. These species were identified as *A. fumigatus* before molecular biological techniques were used in taxonomic studies. However, presently, *A. fumigatus*-related species comprise several species different from *A. fumigatus* based on polyphasic taxonomy including DNA sequences[4)]. This taxon was not recognized as an important causative agent of aspergillosis until recently, but the situation has changed with the report of

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species that have shown resistance to azole antifungal agents[5]. The species generally classified as *A. fumigatus*-related are *A. lentulus* of ser. *Fumigati*; *Aspergillus felis*, *Aspergillus pseudoviridinutans*, *A. udagawae*, *Aspergillus viridinutans*, and *Aspergillus wyomingensis* of ser. *Viridinutantes*; *A. hiratsukae* of ser. *Unilaterales*; and *A. thermomutatus* of ser. *Thermomutati*[4, 6-14]. In Japan, *A. hiratsukae*, *A. lentulus*, *A. viridinutans*, and *A. udagawae* have been isolated from clinical samples[13, 15]. Approximately 3–6% of cases with severe invasive aspergillosis are attributed to the *A. fumigatus*-related species [16-18].

Aspergillus sect. *Fumigati* has been frequently isolated from the soil and large animals, including humans[19], but its distribution patterns in the outdoor environment are not well understood. *A. fumigatus* is more abundant in mid-latitude forests based on global surveys of the distribution of *Aspergillus* species[20]. However, *A. fumigatus*-related species have only been inferred to be capable of a wide distribution, based on limited reports of the source of isolation. The sources of isolates of each species are summarized as follows: *A. felis* from coal mine accumulations in USA and forest soils in Australia, *A. lentulus* from corn and pepper field soils in Korea and soil after herbicide application in Australia, *A. pseudoviridinutans* from Indian and Brazilian soils, *A. udagawae* from grassland soils in USA and plantation soils in Brazil, and *A. wyomingensis* from coal mine accumulations in USA and Russian soils[21-23]. As the information based on these isolation reports is fragmentary, the distribution characteristics of each species in outdoor environments are still unknown. Watanabe et al.[24] recently revealed that *A. felis*, *A. pseudoviridinutans*, *A. lentulus*, *A. udagawae*, and *A. wyomingensis* are commonly distributed in the outdoor environment in Japan and discussed that these species tend to be more abundant in bare land where vegetation is scarce and in farmland. However, the distribution characteristics of each species have not been quantitatively evaluated by Watanabe et al.[24] and remain poorly understood.

In this study, we quantitatively evaluated the distribution of *Aspergillus* sect. *Fumigati* in the outdoor environment of Izu and Ogasawara Islands. In particular, we clarified the species composition and frequency of occurrence of *Aspergillus* sect. *Fumigati* in bare land, grassland, and forests on nine islands and compared the data among islands and different landscapes to elucidate the distribution characteristics of each species. Izu and Ogasawara Islands have never been connected to mainland Japan (Honshu). These islands have a gradient in distance from Honshu, and many of the islands have diverse landscapes related to vegetational succession within a small area owing to the effect of volcanoes. We considered Izu and Ogasawara Islands to be a suitable field for studying the characteristics of the present distribution of *Aspergillus* species and the processes that shaped their distribution.

Materials and methods

Soil sampling and fungal isolation

Izu and Ogasawara Islands are oceanic islands located to the south of central Tokyo. The islands studied were, in order of proximity to central Tokyo, Izu-oshima (A), Toshima (B), Shikinejima (C), Kozushima (D), Miyakejima (E), Hachijo-jima (F), Mukojima (G), Chichijima (H), and Hahajima (I) (A-F: Izu Islands, G-I: Ogasawara Islands) (Fig. 1). Eight to seventeen soil samples were collected between October 2017 and March 2020 at 68 sites, which included as many different landscapes as possible within each island (Table 1). To evaluate the effect of sampling season on isolated fungal species and frequency of occurrence, we sampled ten soils every 2 months from September 2019 to July 2020 at two sites on Miyakejima (E), a grassland site dominated by *Fallopia japonica* var. *hachidyoensis* and *Miscanthus sinensis* var. *condensatus* (E3) and a forest site dominated by *Castanopsis cuspidata* var. *sieboldii* (E7)[25]. Twenty to 40 g of soil sampled at each site was placed in a sterilized culture bottle, and then ten sterilized corn grains were placed on top of the soil and incubated at 35℃ for 7–10 days. After incubation, the conidia of *Aspergillus* species, which appeared on corn grains, were isolated under a stereomicroscope. The detailed methods of soil sampling and fungal isolation from soil samples were as described by Watanabe et al.[24].

Molecular analysis and species identification

DNA from each strain was extracted from the mycelium cultured on malt extract agar overlaid with cellophane membrane using either the cetyl trimethyl ammonium bromide method[26] or the benzyl chloride method[27]. Polymerase chain reaction (PCR) for the calmodulin gene (*CaM*) was performed using the PCR primer pair CMD5/CMD6[28]. The PCR reaction mixture was prepared by adding 21 µL Quick Taq HS DyeMix (Toyobo, Osaka, Japan), 25 µL sterile Milli-Q water, 25 µM forward primer, 25 µM reverse primer, and 3 µL DNA extraction solution. DNA fragments were amplified using a PCR thermal cycler (DNA Engine; Bio-Rad, Hercules, California, USA) with the following thermal cycling schedule: first cycle of 2 min at 94℃, followed by 35 cycles of 30 s at 94℃, 30 s at 54℃ for annealing, and 1 min at 68℃, with a final cycle of 10 min at 68℃. The amplified product was purified using the Fast Gene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). Purified DNA was sequenced by Fasmac (Kanagawa, Japan). A BLAST search of GenBank was performed using the newly obtained sequences to identify closely related species. The sequences were deposited in the DNA Data Bank of Japan (DDBJ; LC702914–LC703521). In addition, morphological observations were made following Samson et al.[19, 29] and Houbraken et al.[1] for species identification of each strain.

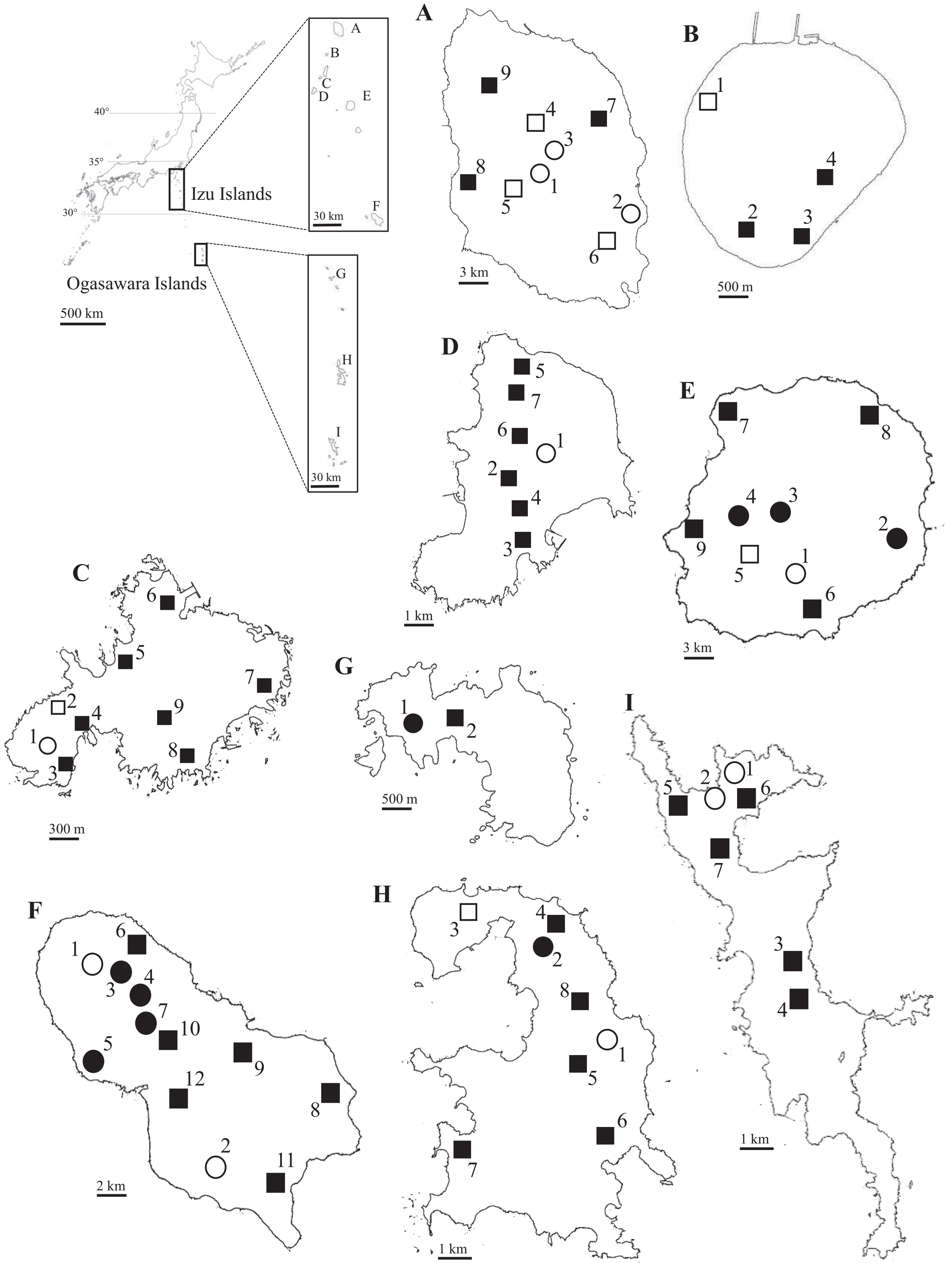

Fig. 1. Soil sampling sites in Izu and Ogasawara Islands.

A: Izu-oshima, B: Toshima, C: Shikinejima, D: Kozushima, E: Miyakejima, F: Hachijojima, G: Mukojima, H: Chichijima, I: Hahajima. Open circle represents bare land, closed circle represents grassland, open rectangle represents shrub forests, and closed rectangle represents forest.

Table 1. Sampling sites in Izu and Ogasawara Islands

Island	Sampling month	Site name	Landscape	Number of soil samples	Latitude	Longitude	Altitude (m)	Mean annual temperature (℃)
Izu-oshima	2018. 9	A1	Bare land	17	34.73869444	139.4033333	510.0	13.8
		A2	Bare land	15	34.73002778	139.4333889	346.0	14.8
		A3	Grassland	15	34.74086111	139.4028056	493.0	13.9
		A4	Shrub forest	15	34.74977778	139.3998056	483.0	13.9
		A5	Shrub forest	15	34.73575	139.38	541.0	13.6
		A6	Shrub forest	15	34.73002778	139.4333889	346.0	14.8
		A7	Forest	15	34.76333333	139.4299444	209.0	15.6
		A8	Forest	15	34.73334167	139.3591944	60.0	16.5
		A9	Forest	15	34.77080556	139.3600278	117.0	16.1
Toshima	2019. 11	B1	Shrub forest	14	34.521541	139.26908	161.9	16.8
		B2	Forest	15	34.515857	139.274014	271.9	16.1
		B3	Forest	15	34.515583	139.277191	287.9	16.0
		B4	Forest	15	34.521122	139.285061	288.5	16.0
Shikinejima	2019. 11	C1	Bare land	15	34.321256	139.196841	88.6	17.2
		C2	Shrub forest	15	34.325316	139.203091	88.8	17.2
		C3	Forest	15	34.320748	139.201973	78.0	17.3
		C4	Forest	15	34.32287	139.202772	74.9	17.3
		C5	Forest	15	34.3283	139.208252	55.2	17.4
		C6	Forest	15	34.333963	139.213411	42.4	17.5
		C7	Forest	15	34.32531	139.224913	47.0	17.5
		C8	Forest	15	34.319257	139.21213	31.8	17.6
		C9	Forest	15	34.322586	139.210307	46.7	17.5
Kozushima	2017. 10	D1	Bare land	12	34.21839324	139.1569405	507.2	15
		D2	Forest	12	34.2144545	139.1536005	484.4	15.1
		D3	Forest	12	34.19594332	139.1536964	75.1	17.6
		D4	Forest	12	34.20717666	139.1536964	143.6	17.2
		D5	Forest	12	34.24068742	139.1536964	55.6	17.7
		D6	Forest	12	34.22225914	139.1536964	19.4	17.9
		D7	Forest	12	34.23086031	139.1536964	208.5	16.8
Miyakejima	2018. 6	E1	Bare land	16	34.07075	139.5098333	459.0	15.5
		E2	Grassland	15	34.07947222	139.5548056	115.0	17.5
		E3	Grassland	15	34.078	139.5164444	536.0	15.0
		E4	Grassland	15	34.07497222	139.5072778	413.0	15.8
		E5	Shrub forest	15	34.07991667	139.5038333	385.0	15.9
		E6	Forest	15	34.0565	139.5233611	28.0	18.1
		E7	Forest	15	34.11208333	139.5016389	118.0	17.5
		E8	Forest	15	34.10769444	139.5580278	17.0	18.1
		E9	Forest	15	34.08180556	139.4856944	74.0	17.8
Hachijojima	2019. 6	F1	Bare land	15	33.140721	139.762634	832.1	13.9
		F2	Bare land	15	33.072318	139.783907	194.6	17.7
		F3	Grassland	15	33.139904	139.7672	825.9	14
		F4	Grassland	15	33.140939	139.762868	823.5	14
		F5	Grassland	16	33.105999	139.755304	20.8	18.8
		F6	Forest	15	33.13764	139.770338	672.6	14.9

		F7	Forest	15	33.128044	139.780881	224.6	17.6
		F8	Forest	15	33.089439	139.847957	172.4	17.9
		F9	Forest	15	33.099759	139.836321	452.4	16.2
		F10	Forest	15	33.111291	139.791309	99.1	18.3
		F11	Forest	15	33.064921	139.816947	133.3	18.1
		F12	Forest	15	33.095329	139.800707	386.3	16.6
Mukojima	2020. 3	G1	Grassland	15	27.681839	142.129	5	23.4
		G2	Forest	15	27.682471	142.129719	18	23.3
Chichijima	2019. 3	H1	Bare land	12	27.074881	142.224385	244.5	21.9
		H2	Grassland	12	27.094659	142.20785	279.6	21.7
		H3	Shrub forest	12	27.102421	142.206668	116	22.7
		H4	Forest	12	27.095241	142.208651	260.9	21.9
		H5	Forest	12	27.059214	142.224989	283	21.7
		H6	Forest	12	27.059811	142.225201	266.9	21.8
		H7	Forest	12	27.059474	142.196144	57.1	23.1
		H8	Forest	12	27.074046	142.222309	328.7	21.4
Hahajima	2020. 2	I1	Bare land	8	26.699415	142.149345	294.3	21.7
		I2	Bare land	10	26.698574	142.148908	264	21.8
		I3	Forest	15	26.658086	142.159597	395	21
		I4	Forest	10	26.650654	142.159908	209.7	21.4
		I5	Forest	11	26.699698	142.142902	64.3	23
		I6	Forest	15	26.701543	142.141916	46	23.1
		I7	Forest	15	26.698995	142.149181	280.6	21.7
		I8	Forest	15	26.698712	142.148979	277.2	21.8

Evaluation of distribution of each species at each sampling site

The frequency of occurrence was calculated to evaluate the distribution of *Aspergillus* sect. *Fumigati* at each sampling site by using the following equation:

Frequency of occurrence (%) = (number of soil samples from which a species was isolated/number of soil samples collected [n = 8–17]) × 100

Results

Seasonal changes in the frequencies of occurrence at two Miyakejima sampling sites

Aspergillus sect. *Fumigati* (three species: *A. fumigatus*, *A. pseudoviridinutans*, and *A. udagawae*) were isolated at grassland E3 and forest E7 sites in Miyakejima, and the seasonal frequencies of occurrence at the two sampling sites were compared. At E3, only *A. pseudoviridinutans*, but not *A. fumigatus* or other sections, was isolated. At E7, *A. fumigatus* and *A. udagawae* were isolated. At E3, *A. pseudoviridinutans* was isolated at high frequencies (90–100%) throughout the year (Fig. 2). At E7, *A. udagawae* occurred at a low frequency (10%) in September, November, January, and July but was not isolated in the other sampling months (Fig. 2). At the same site, *A. fumigatus* occurred at variable frequencies (10–60%) in all sampling months, except November (Fig. 2). The other sections did not occur in November and June; however, in the other sampling months, they occurred at frequencies of 10–40% (Fig. 2).

Distribution pattern in Izu and Ogasawara Islands

Isolates of *Aspergillus* sect. *Fumigati* from 68 sampling sites on Izu and Ogasawara Islands were identified and the frequencies of occurrence were calculated: *A. pseudoviridinutans* (495 strains), *A. fumigatus* (351 strains), *A. felis* (311 strains), *A. udagawae* (164 strains), *Aspergillus arcoverdensis* (22 strains), *A. lentulus* (4 strains), and *Aspergillus spinosus* (1 strain). The frequency of occurrence of *A. pseudoviridinutans*, *A. fumigatus*, *A. felis*, and *A. udagawae*, which had the highest number of strains isolated, at each sampling site is separately summarized for bare land and grassland (Fig. 3A) and for shrub forests and forests (Fig. 3B). In a total of 19 sites with bare land and grassland landscapes, *A. fumigatus* was isolated at 5 sampling sites on Izu-oshima (A), Shikinejima (C), Kozushima (D), Miyakejima (E), and Hachijojima (F) with frequencies ranging from 6% to 17%; *A. felis* at 6 sites on Izu-oshima (A), Shikinejima (C), Miyakejima (E), and Hachijo-

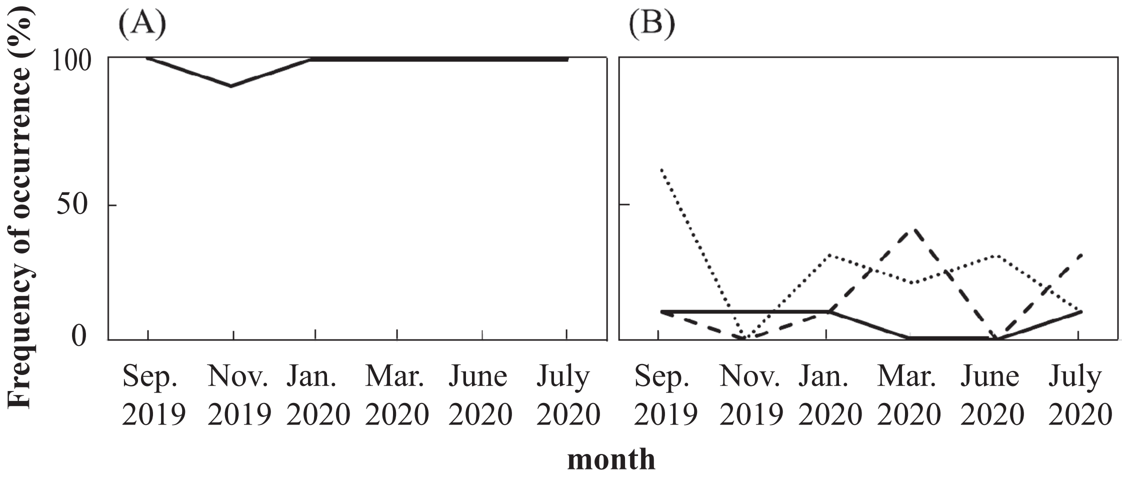

Fig. 2. Seasonal variation in the frequency of occurrence of *Aspergillus* section *Fumigati* at grassland E3 (A) and forest E7 (B) sites in Miyakejima.

Solid lines represent *Aspergillus pseudoviridinutans* (A) and *Aspergillus udagawae* (B), dotted line represents *A. fumigatus*, and dashed line represents other *Aspergillus* species.

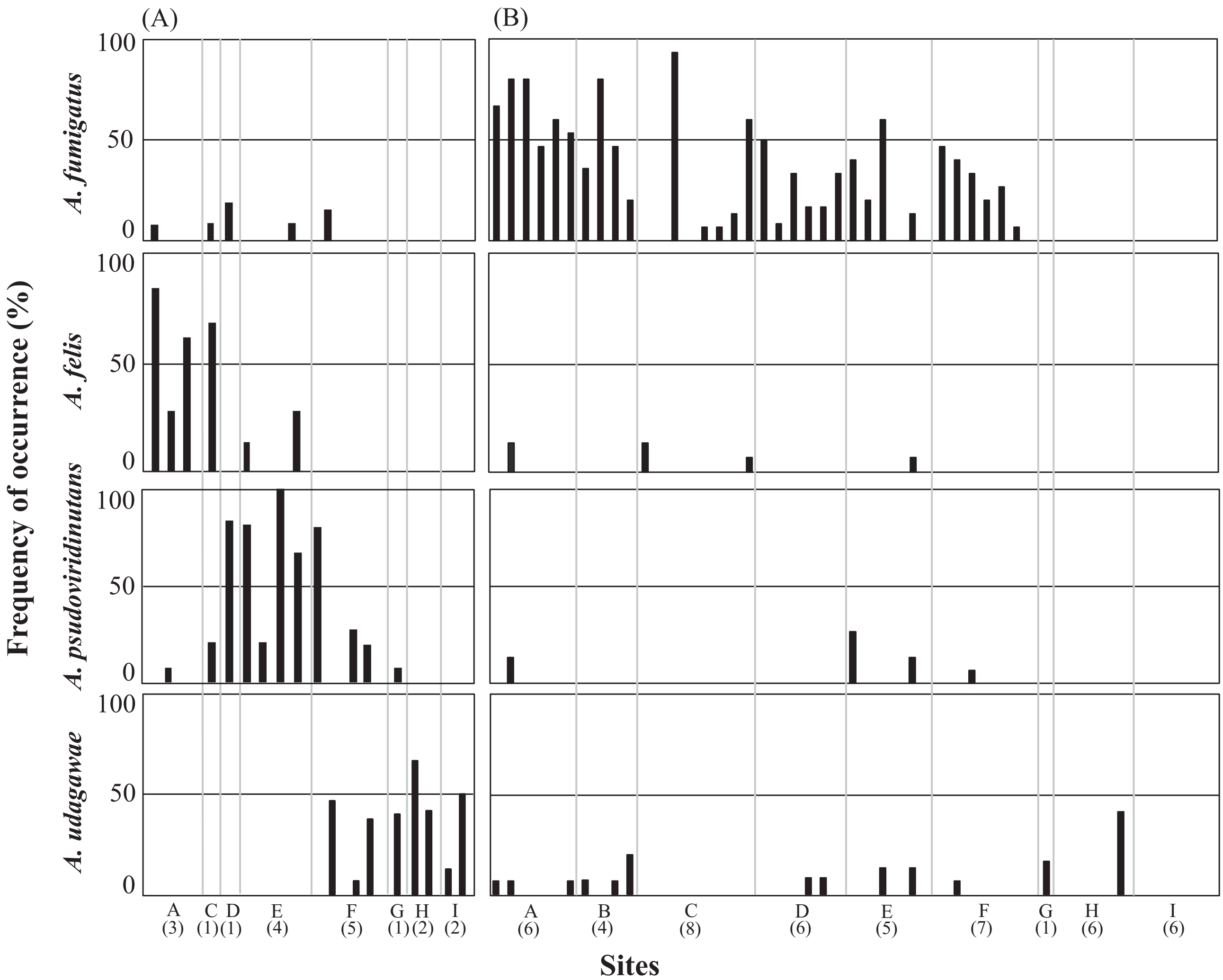

Fig. 3. Frequency of occurrence of *Aspergillus* section *Fumigati* at each site in Izu and Ogasawara Islands.

A: Bare land and grassland, B: shrub forest and forest. The number of sampling sites on each island is represented in parentheses below the island name.

jima (F) with 13−82% frequency; *A. pseudoviridinutans* at 11 sites on Izu-oshima (A), Shikinejima (C), and Hachijojima (F) with 7−100% frequency; *A. udagawae* at 8 sites on Hachijojima (F), Chichijima (H) and Hahajima (I) with 6−67% frequency; and *A. arcoverdensis* at 2 sites on Chichijima (H) and Hahajima (I) with 10−17% frequency; *A. lentulus* at 1 site on Hachijojima (F) with 13% frequency (Fig. 3). By contrast, in a total of 49 sites with shrub forest and forest landscapes, *A. fumigatus* was isolated at 31 sites on 6 islands from Izu-oshima (A) to Hachijojima (F) at frequencies ranging from 7% to 93%; *A. pseudoviridinutans* at 4 sites on Izu-oshima (A), Miyakejima (E), and Hachijojima (F) with 7−27% frequency; *A. udagawae* at 13 sites on Izu-oshima (A), Toshima (B), Kozushima (D), Miyakejima (E), Mokujima (G), and Chichijima (H) with 6−40% frequency; *A. arcoverdensis* at 1 site on Chichijima (H) with 8% frequency; and *A. lentulus* at 1 site on Shikinejima (C) with 7% frequency; *A. spinosus* at 1 site on Toshima (B) with 7% frequency (Fig. 3).

These results indicate that the frequency of occurrence of *A. fumigatus*-related species was high in bare land and grassland, whereas that of *A. fumigatus* was high in shrub forests and forests. This pattern was common to all the islands. *A. fumigatus* was more frequent between Izu-oshima (A) and Shikinejima (C), less frequent on islands south of these islands, and not isolated on Ogasawara Islands. *A. felis* was more frequent on Izu-oshima (A) and Shikinejima (C), *A. pseudoviridinutans* on Kozushima (D) and Hachijojima (F), and *A. udagawae* on Mukojima (G) and Hahajima (I) (Fig. 3). No sampling sites were dominated by *A. arcoverdensis*, *A. lentulus*, or *A. spinosus*.

Discussion

A year-long survey of the diversity of *Aspergillus* sect. *Fumigati* on Miyakejima (E) revealed that the frequency of occurrence of *A. pseudoviridinutans*, one of the *A. fumigatus*-related species, was extremely high throughout the year at E3. No other *Aspergillus* species capable of growing at 35℃ were isolated from this site. The environment at E3 is characterized by three factors: first, summation of vegetation coverage, which was calculated as the sum of total coverage of all vegetation species, is lower in E3 (25%) than in E7 (135%) (Hirota, unpublished data), which makes the soil surface more exposed to UV radiation; second, low vegetation cover causes severe soil surface drying[30]; and third, carbon sources are scarce (C/N = 5.6) (Hirota, unpublished data). There are few reports of *A. fumigatus*-related species isolated in such harsh environments, although *A. wyomingensis* has been isolated from coal mine soils[23]. To elucidate why *A. pseudoviridinutans* can survive year-round under such harsh environmental conditions, it is necessary to clarify the physiological characteristics of cultured strains. By contrast, *A. fumigatus*, which was isolated only at E3, showed a wide range in its frequency of occurrence depending on the season of soil sampling (Fig. 2). This is in contrast with the result of another study that showed no seasonality in the amount of airborne *A. fumigatus*[31]. In particular, the absence of *A. fumigatus* in November could be due to the occurrence of fungal species that grow more rapidly than *Aspergillus* species at an incubation temperature of 35℃. If such fungal species occurred, the frequency of occurrence of the target fungus would be underestimated. In this study, the occurrence of such fungi was not confirmed at other sampling sites.

Seven species of *Aspergillus* sect. *Fumigati* were found on nine islands in Izu and Ogasawara Islands. Of these species, *A. fumigatus* was abundant in shrub forests and forests on all islands, except Ogasawara Islands (Fig. 3). *A. fumigatus* tends to be distributed in mid-latitude forests[20], and the results of this study support this observation. The distribution of *A. fumigatus* was not confirmed at forest landscape sites in Ogasawara Islands because long-distance dispersal by spores could be limited, even though the distribution range of this species is quite wide[20]. *A. felis*, *A. pseudoviridinutans*, and *A. udagawae*, which are *A. fumigatus*-related species, were isolated from more sites than the other three species, which allowed us to characterize their distribution in Izu and Ogasawara Islands. The frequency of occurrence of these three species was higher in bare land and grassland than in forests, regardless of the island (Fig. 3). Of the three species, *A. udagawae* was found to differ from the other two species in that it is widely distributed in the forest landscape, although its frequency of occurrence is not high. Watanabe et al.[24] noted that *A. pseudoviridinutans* and *A. udagawae* tend to be frequently isolated from grasslands and farmlands, consistent with the results of this study. It is clear that even within the same island, different species are abundant in different landscapes possibly owing to the physiological characteristics of each species.

One common species of the three *A. fumigatus*-related species was isolated at a high frequency, regardless of the soil sampling site, within the same island. *A. felis* was frequently isolated from Izu-oshima (A) to Shikinejima (C), *A. pseudoviridinutans* from Kozushima (D) to Hachijojima (F), and *A. udagawae* from the Ogasawara Islands (Fig. 3). Islands with the same dominant species are relatively close in geographic distance, suggesting that spore dispersal may occur frequently among the islands. Between Izu-oshima (A) and Shikinejima (C), spore dispersal from Honshu, the main island of Japan, is strongly affected. By contrast, the species occurring between Kozushima (D) and Hachijojima (F) and on Ogasawara Islands may have spread their distribution area to the surrounding islands after accidental establishment via the westerlies and ocean.

The number of isolates and sampling sites of three species, *A. arcoverdensis*, *A. lentulus*, and *A. spinosus*, were lower than those of the other *Aspergillus* sect. *Fumigati* species; therefore, it was not possible to characterize their distribution in Izu and Ogasawara Islands. *A. arcoverdensis*, from Ogasawara Islands, has been previously isolated from semi-desert soils[32]. In the present study, this species was isolated from both bare land (frequency of occurrence = 10−17%) and forest including shrub forest (8%), suggesting that it may be widely distributed in Ogasawara Islands regardless of the landscape. Similarly, *A. lentulus* was reported to be isolated from plant residues and farmland[33], but in the present study, this species was isolated from grassland (13%) on Hachijojima and forest (7%) on Shikinejima, suggesting that it may also be widely distributed regardless of landscape. *A. spinosus* was reported to be isolated from farmland and plant residues[28]; however, in this study, this species was isolated only from a shrub forest (7%) on Toshima. Further collection of strains and accumulation of environmental data on the isolation source are warranted to clarify the distribution characteristics of these fungi.

The distribution characteristics of *Aspergillus* sect. *Fumigati*, which is frequently isolated from the soil in Izu and Ogasawara Islands, were clarified in this study. *A. felis*, *A. pseudoviridinutans*, and *A. udagawae* are widely distributed in bare land and grassland, regardless of the islands, and the high-frequency species varied depending on the location of the islands. *A. fumigatus* was widely distributed in the forest landscape of the islands, except on Ogasawara Islands. In the future, we will conduct population genetic studies to determine genetic differentiation among islands for each species and to elucidate the spore dispersal process of these species.

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Transparency declarations

All authors: none to declare.

Conflicts of interest

Self-declared COI content: None.

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