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Original

Ochroconis Species Isolated from Limestone Areas as the Origin of Indoor *Ochroconis*

NOBUO HAMADA[1]*, AND NIICHIRO ABE[2]

[1]*Osaka Museum of Natural History, 1-23 Nagai Park, Higashi-Sumiyoshi, Osaka 546-0034, Japan*
[2]*Osaka Institute of Public Health, 8-34 Tojo-cho, Tennoji, Osaka 543-0026, Japan*

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The molecular, morphological, and physiological features of 42 strains of *Ochroconis* collected from many limestone areas were studied. *Ochroconis* strains are often found in limestone areas, although they have rarely been found in other wild areas, e. g. forests. Moreover, many strains from these areas grew on alkaline media (pH 9.7) and media with soap. They were phylogenetically more variable than *Ochroconis* strains previously found indoors or at city parks. Thus, the *Ochroconis* strains are thought to have adapted to the alkaline soil, specifically found in limestones. It is assumed that some strains of *Ochroconis* originally grew in the limestones and immigrated into park soils with the fine dust of cement and into indoor environments. More species of *Ochroconis*, with the ability to use surfactants as nutrients, are distributed in limestone areas than indoors or in city parks. Moreover, these fungi were found randomly in the phylogenetic trees of *Ochroconis*. Although only *O. humicola* was often found indoors and used surfactants, this fungus was newly found in the limestone areas of Okinawa Prefecture. *Ochroconis humicola* originally grew outdoors and seems to have immigrated to and propagated indoors. Thus, this species may have originated from the subtropical limestone areas.

Key words : Alkaline condition / Detergent / Drought / Heat tolerance / Limestone.

INTRODUCTION

Some unique fungi, using detergents as nutrients, have been found in water supply environments (Hamada, 2002; Hamada and Abe, 2009). Previous studies showed that *Ochroconis humicola*, using non-ion surfactants as nutrients, was predominantly found in indoor environments (Abe and Hamada, 2011) and distributed across Asia, Europe, and USA. *Ochroconis humicola* may not have grown indoors until about 50 years ago, when the custom of using detergents in the water supply began (JSWE, 2000).

Since these fungi may have immigrated from specific outdoor environments, we examined environments where *O. humicola* may have grown originally. We tried to collect *Ochroconis* strains from rural outdoor environments, such as mountains, forests, and agricultural fields. However, it was difficult to collect not only *O. humicola*, but also some other species of *Ochroconis* (Hamada and Abe, 2018). Some strains of *Ochroconis* were found in the superficial soils of urban city parks, on which the fine dust of cement slightly accumulates, making it more alkaline than superficial soils in forests and fields. An *Ochroconis* strain, designated as Group P, was found in city parks and was physiologically similar to the indoor species, *O. humicola*. Group P can use detergents as nutrients and grows under alkaline conditions. However, *O. humicola* was not found in the city parks of the Honshu and Kyushu Islands (Hamada and Abe, 2018).

If alkaline soil promotes the growth of *Ochroconis* strains in the parks, it can be assumed that many species of *Ochroconis* were distributed in limestone areas and immigrated to other alkaline environments. Moreover, some of them may be able to grow on media containing non-ion surfactants.

In a preliminary study, we collected the soils from

*Corresponding author. Tel: +81-90-7757-1087, Fax: +81-6-6697-6225, E-mail : mxi00715(a)nifty.com

regions outside and inside of limestone caves and isolated several *Ochroconis* strains. In this study, phylogenetic analysis, morphological observation, and culture examination, using media including detergents or others, were conducted on samples from various localities. *Ochroconis* strains from the soils of limestone areas were also compared with strains from indoors or city parks, by focusing on alkaline tolerance, heat resistance, and drought tolerance. Moreover, factors controlling the growth of *Ochroconis* strains under three different environmental conditions, namely indoors, in city parks, and in limestone areas, were also examined. The immigration process of *Ochroconis* strains from limestone areas to indoors or city parks was also discussed.

In this study, the genus *Ochroconis* was used in place of *Scolecobasidium*, according to previous reports (de Hoog et al., 2000; Samerpitak et al., 2014).

MATERIALS AND METHODS

Collection of samples

To isolate *Ochroconis* strains, we collected soil samples from limestone areas, including inside limestone caves. At each sampling site, about 30 g of superficial soil from the surface layer was collected at 6-12 spots, as previously described for sample collection from city parks (Hamada and Abe, 2018).

Approximately 2 g of collected soil was suspended in 100 mL of distilled water; the suspension was diluted 1:100 and 1:1,000 and then spread onto 1/4 potato dextrose agar (PDA). After incubation for 7–10 d at 25 °C, brown or light brown colonies resembling *Ochroconis* were isolated for identification. One *Ochroconis* strain, among two or more strains with similar features, was selected from each sampling site. In this study, about 50 *Ochroconis* strains were isolated from 26 of the 29 sampling sites in limestone areas (Table 1). Moreover, some strains with few conidia were eliminated from the list of collected samples, because examining their physiological features was difficult.

The pH of the soil samples (10 g) in which the fungi were detected was measured following a standard method (Hamada and Abe, 2018).

Strains and DNA extractions

The 42 *Ochroconis* strains used for this study, together with their localities, are listed in Table 1. Prior to the present study, all isolates were identified as *Ochroconis* at the genus level according to the characteristics of their colonies and conidia (de Hoog et al., 2000). After preculture, a small piece of agar with one colony was added in 1 mL of phosphate buffered saline (PBS) for DNA extraction. The DNA was extracted and purified using the DNeasy Blood & Tissue kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) and phylogenetic analysis

Previously, our study showed that the phylogenetic topologies reconstructed using the sequences of 18S rDNA, 28S rDNA, *tef1*, or *rbp2* were the same among *Ochroconis* spp. (Abe and Hamada, 2011). The 28S rDNA locus was applied for the present analysis. A phylogenetic analysis was carried out using the MEGA7 software. The 28S rDNA sequences obtained were aligned with those from representative *Ochroconis* species. A phylogenetic tree was reconstructed using the neighbor-joining method with the p-distance algorithm. The reliability of the phylogram was tested with the bootstrap method using 1,000 replications. The 28S rDNA sequences obtained in this study are available in the International Nucleotide Sequence Database (GenBank/DDBJ/EMBL) under accession numbers LC469356-LC469397.

Morphological examination

For each strain, the color, size, shape, wall structure, and number of cells comprising conidia in each colony were observed by a light microscope (ECLIPSE E600; Nikon, Tokyo, Japan), and by magnifying the images 1,000 times by oil immersion light microscopy (Leica, Germany).

Growth of *Ochroconis* species on various media

The 42 precultured outdoor strains were examined for their physiological characteristics, as described previously (Hamada and Abe, 2018). A 0.5 mL of the conidial suspension from each strain was plated uniformly on two culture plates containing media with different compositions (see below). All plates were incubated at 25 ± 1 °C for 10–12 d. The fungal count shown in Table 2 is presented as an average of the colony numbers on two plates.

The growth of *Ochroconis* strains was examined on media containing two surfactants: soap, sodium oleate (SO), or non-ion surfactant, polyoxyethylene-(9)-lauryl ether (AE). To prepare these media, SO or AE at 0.1% w/v was added to 15 g/L of Bacto agar (Difco, USA).

Each strain was cultured on the alkaline 1/4 PDA medium to examine its tolerance to alkaline conditions. To adjust the pH value of the medium to an alkaline level, a modified version of the method reported by Nagai et al. (1998) was applied. Namely, Na_2CO_3 (3 g) and NaH_2PO_4 (3 g) were added to 1/4 PDA. The pH of 1/4 PDA was 6.6, whereas that of the modified (*i.e.*, alkaline) 1/4 PDA was 9.7.

To examine the tolerance of the fungi to drought, each

strain was cultured on dichloran glycerol (DG18) agar base (Oxoid, England) containing 31.5 g/L and 220 g/L glycerol, with the latter concentration being used to detect xerophilic fungi.

To examine the heat stress tolerance of fungi, conidial suspensions of each fungus were placed in water baths (Masuda; M-205) at 50 ± 1 °C for 10 min. After treatment, the suspensions were inoculated and cultured onto 1/4 PDA.

RESULTS

Molecular characterization of the strains

The 28S rDNA sequences were obtained from 42 strains of *Ochroconis* collected from limestone areas from Iwate Pref. (40N) to Okinawa Pref. (24N). Some strains were identified genetically by comparison with ones collected from indoors or city parks in previous studies (Hamada and Abe, 2018). As shown in Fig. 1, the two strains (NH2012 and NH754) from Okinawa Pref. were classified into *O. humicola*, which was predominantly found indoors. The eight strains isolated from the limestone areas and the predominant strains of *Ochroconis* (NH503) found in urban city parks were grouped together (park group: Group P). But Group P was more common in city parks, where 14 out of 24 *Ochroconis* strains were found. Moreover, three strains were identical to ones from city parks; NH552 was identified as *O. constricta*, NH236 as *O. verruculosa*, and NH273 as *Ochroconis* sp. NH11772. The 42 strains collected from the limestone areas varied more than strains from city parks.

The other 29 strains were not found in city parks. Some of them were identified as species reported previously (Samperpitak et al., 2009). Three strains of *O. macrozamiae*, three of *O. cordanae*, and two of *S. excentricum* were found. Moreover, one strain of *O. lascauxensis, O. anellii, O. globalis, O. bacilliformis*, and *O. robusta* was also found. The other 16 strains of *Ochroconis* species collected were genetically different from each other and were hence thought to be novel.

Ochroconis strains were found both inside and outside the caves in the limestone areas, although the fungal number found inside the caves was less than 1/10th of that found outside.

Morphological characterization of the strains

The conidia of two strains of isolated *O. humicola* were two-celled, pale brown, cylindrical-shaped, 8–9 µm in length, and smooth-walled or verruculose (Table 1). The conidia of eight strains of Group P were two-celled, pale brown, cylindrical-shaped, 7–9 µm in length, and verrucose or echinulate-walled.

The NH236 strain showed two-celled, oblong, and echinulate-walled conidia, and resembled those of *O. verruculosa*. Moreover, the conidia of NH552 were similar to those of *O. constricta*. The other strains, for example *O. cordanae* and *O. macrozamiae*, which were identified as species previously reported, were confirmed morphologically.

The pH of each soil sample from the limestone area was analyzed (Table 1). The pH ranged from 6.09 to 9.33. The average pH was 7.57 and was more alkaline than the pH of 6.89 found in city parks. The average pH inside the caves was 8.30, relatively more alkaline than the pH of 7.37 found outside the caves.

Growth differences among Ochroconis species

Most of the isolated strains (36/42) grew on 0.1% SO medium, and the colony number for most strains (32/36) was ≥50% of those grown on 1/4 PDA (control), although the size of the colonies was smaller than that on 1/4 PDA (Table 2).

Only 13 *Ochroconis* strains grew on the 0.1% AE medium. Interestingly, two strains of *O. humicola*, four to eight strains of Group P, and one strain of *O. constricta* grew on the 0.1% AE medium as well as strains from city parks or indoors. Otherwise, 6 to 31 other strains of *Ochroconis* grew on the 0.1% AE medium; two strains of *O. cordanae*, and a strain of *O. verruculosa* grew on them, and 3 strains of *Ochroconis* sp., which was different in phylogenetic analysis, grew on them.

On the 1/4 PDA medium adjusted to pH 9.7, 34/42 isolated strains grew. All of the *O. humicola* and Group P strains grew on the alkaline medium. Moreover, all strains able to use 0.1% AE grew on the alkaline medium. The size of the colonies on alkaline medium was similar in 10 of 42 strains, compared to those growing on the control.

The drought tolerance of *Ochroconis* strains was compared. Some strains (14/42) of *Ochroconis* samples grew on the xerophilic medium (DG18), although the colony size for all these strains was smaller than colonies growing on the control medium. Moreover, seven strains, including *O. humicola, O. constricta, O. cordanae,* and *O. verruculosa*, growing on xerophilic medium also grew on the AE medium.

The heat tolerance of the strains was analyzed according to the incubation temperature (*i.e.*, 50 °C) (Table 2). More than half of the *Ochroconis* strains (25/42) could survive at 50 °C, with 13 to 15 strains belonging to *O. humicola,* Group P, *O. cordanae*, *O. constricta*, and *O. verruculosa*, including strains growing on the AE medium. Most AE-positive strains (12/13) exhibited heat tolerance. In addition, strains growing on 0.1% AE media, including *O. humicola* and Group P strains, showed similar physiological responses to drought and heat.

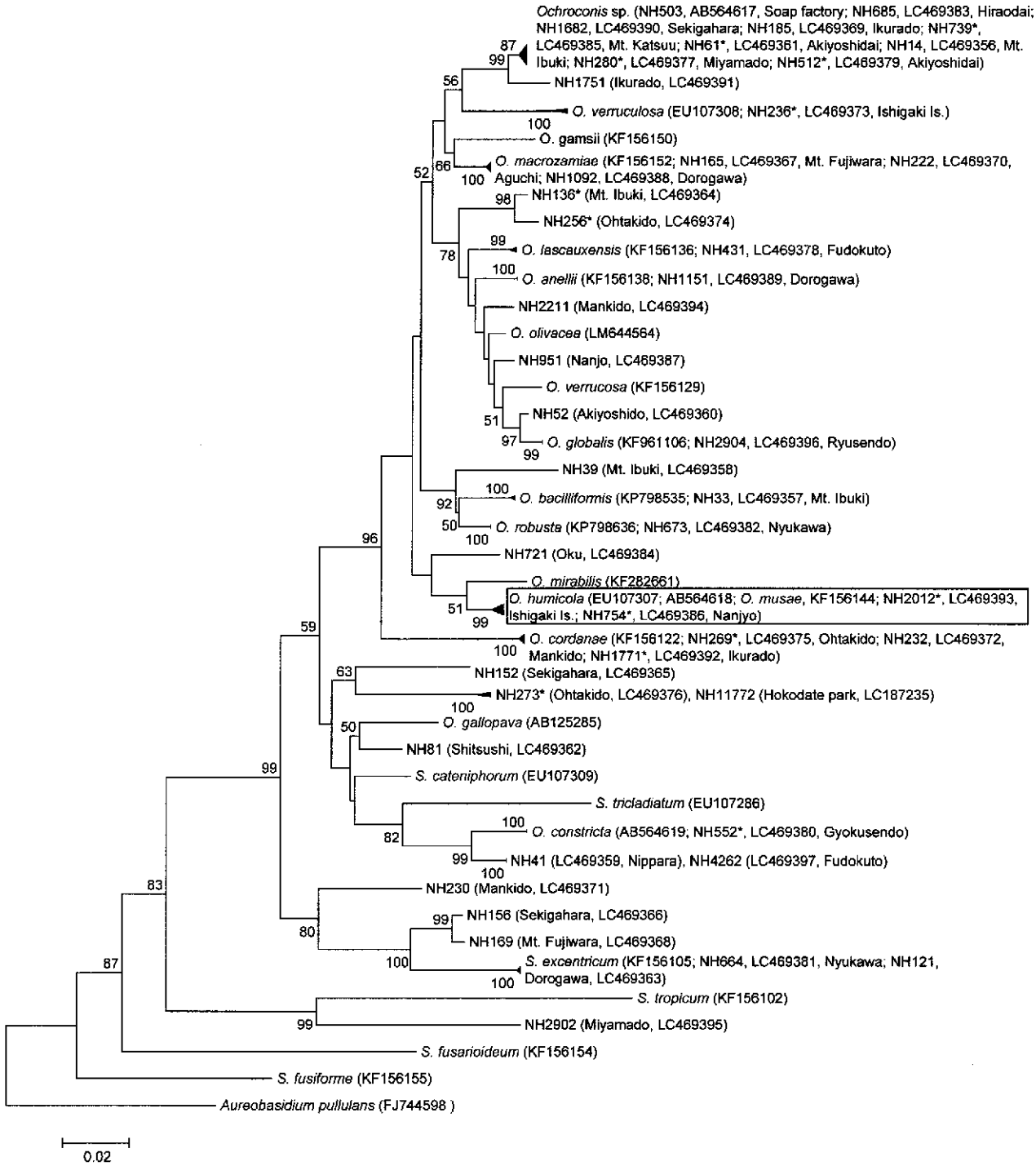

FIG. 1. Phylogenetic relationships of 42 *Ochroconis* strains collected from various localities of limestone areas compared to reference strains of *Ochroconis* species, as inferred from distance-based analysis of 28S rDNA sequences.
The strain number (NH) and accession numbers are shown in parentheses, and the asterisk indicates a strain with the ability to use AE (non-ion surfactant) as a nutrient. Bootstrap support (>50% for 1000 replicates) is shown at each node.

TABLE 1. Fungal characteristics of *Ochroconis* collected from soil of the limestone area

Sample number	Accession number	Species	Group	Locality	Prefecture	Latitude	Altitude	Caves	pH	Conidia				
										Color	Shape	Size	Cell	Wall
754	LC469386	*O. humicola*		Nanjyo	Okinawa	26	50	outside	7.20	pale brown	cylindrical	9	2	smooth
2012	LC469393	*O. humicola*		Ishigaki Is.	Okinawa	24	10	outside	8.08	pale brown	cylindrical	8	2	verruculose
552	LC469380	*O. constricta*		Gyokusendo	Okinawa	26	0	inside	8.04	pale brown	cylindrical	8	2	verrucose
1151	LC469389	*O. anellii*		Dorogawa	Nara	34	850	inside	8.15	pale brown	cylindrical	15	4	smooth
33	LC469357	*O. bacilliformis*		Mt. Ibuki	Shiga	35	1300	outside	7.17	pale brown	cylindrical	9	2	smooth
269	LC469375	*O. cordanae*		Ohtakido	Gifu	36	400	outside	6.99	pale brown	cylindrical	13	2-4	smooth
232	LC469372	*O. cordanae*		Mankido	Okayama	35	300	outside	8.04	pale brown	cylindrical	12	2-4	smooth
1771	LC469392	*O. cordanae*		Ikurado	Okayama	35	200	outside	8.43	pale brown	cylindrical	9	2	smooth
664	LC469381	*S. excentricum*		Nyukawa	Gifu	36	900	outside	8.22	pale brown	cylindrical	12	2	verrucose
121	LC469363	*S. excentricum*		Dorogawa	Nara	34	800	outside	7.12	pale brown	cylindrical	12	2	smooth
2904	LC469396	*O. globalis*		Ryusendo	Iwate	40	10	inside	8.35	pale brown	cylindrical	10	2	smooth
431	LC469378	*O. lascauxensis*		Fudokutsu	Nara	34	600	inside	7.97	pale brown	cylindrical	11	4	verrucose
165	LC469367	*O. macrozamiae*		Mt. Fujiwara	Mie	35	650	outside	6.47	pale brown	cylindrical	6	2	verrucose
222	LC469370	*O. macrozamiae*		Aguchi	Okayama	35	400	inside	7.81	pale brown	cylindrical	9	2	verruculose
1092	LC469388	*O. macrozamiae*		Dorogawa	Nara	34	800	outside	8.36	pale brown	cylindrical	8	2	verruculose
673	LC469382	*O. robusta*		Nyukawa	Gifu	36	900	inside	8.28	pale brown	oblong	7	2	smooth
236	LC469373	*O. verruculosa*		Ishigaki Is.	Okinawa	24	10	outside	8.15	pale brown	oblong	8	2	echinulate
280	LC469377	*Ochroconis* sp.	P	Miyamado	Gifu	36	600	outside	6.64	pale brown	cylindrical	8	2	echinulate
14	LC469356	*Ochroconis* sp.	P	Mt. Ibuki	Shiga	35	900	outside	6.94	pale brown	cylindrical	8	2	verrucose
1682	LC469390	*Ochroconis* sp.	P	Sekigahara	Gifu	35	200	inside	8.06	pale brown	cylindrical	9	2	verrucose
185	LC469369	*Ochroconis* sp.	P	Ikurado	Okayama	35	200	inside	9.33	pale brown	cylindrical	7	2	verrucose
61	LC469361	*Ochroconis* sp.	P	Akiyoshidai	Yamagichi	34	300	outside	7.96	pale brown	cylindrical	8	2	verrucose
512	LC469379	*Ochroconis* sp.	P	Akiyoshidai	Yamagichi	34	300	outside	7.79	pale brown	cylindrical	8	2	echinulate
685	LC469383	*Ochroconis* sp.	P	Hiraodai	Fukuoka	34	400	outside	6.96	pale brown	cylindrical	9	2	verrucose
739	LC469385	*Ochroconis* sp.	P	Mt. Katsuu	Okinawa	27	100	outside	7.15	pale brown	cylindrical	8	2	verrucose
2902	LC469395	*Ochroconis* sp.	–	Miyamado	Gifu	36	600	outside	6.96	subhyaline	clavate	11	2	smooth
41	LC469359	*Ochroconis* sp.	–	Nippara	Tokyo	36	800	outside	7.50	pale brown	cylindrical	7	2	smooth
256	LC469374	*Ochroconis* sp.	–	Ohtakido	Gifu	36	400	inside	8.70	pale brown	cylindrical	12	4	verruculose
273	LC469376	*Ochroconis* sp.	–	Ohtakido	Gifu	36	400	outside	6.09	pale brown	clavate	14	2	smooth
81	LC469362	*Ochroconis* sp.	–	Shitsushi	Kyoto	35	400	outside	6.61	pale brown	cylindrical	10	2	verrucose
39	LC469358	*Ochroconis* sp.	–	Mt. Ibuki	Shiga	35	1300	outside	7.06	pale brown	cylindrical	10	2	smooth
136	LC469364	*Ochroconis* sp.	–	My. Ibuki	Shiga	35	1300	outside	7.31	pale brown	cylindrical	9	2	verrucose
156	LC469366	*Ochroconis* sp.	–	Sekigahara	Gifu	35	200	outside	6.81	pale brown	cylindrical	9	2	smooth
152	LC469365	*Ochroconis* sp.	–	Sekigahara	Gifu	35	200	outside	7.70	pale brown	cylindrical	12	2-4	verrucose
169	LC469368	*Ochroconis* sp.	–	Mt. Fujiwara	Mie	35	800	outside	7.30	pale brown	clavate	15	4	smooth
230	LC469371	*Ochroconis* sp.	–	Mankido	Okayama	35	300	outside	6.70	pale brown	oblong	7	2	verruculose
2211	LC469394	*Ochroconis* sp.	–	Mankido	Okayama	35	300	outside	7.69	pale brown	cylindrical	6	2	verrucose
1751	LC469391	*Ochroconis* sp.	–	Ikurado	Okayama	35	200	outside	7.68	pale brown	cylindrical	8	2	verrucose
4262	LC469397	*Ochroconis* sp.	–	Fudokutsu	Nara	34	600	outside	7.46	pale brown	cylindrical	8	2	verruculose
52	LC469360	*Ochroconis* sp.	–	Akiyoshidai	Yamagichi	34	300	outside	7.49	pale brown	cylindrical	7	2	verrucose
721	LC469384	*Ochroconis* sp.	–	Oku	Okinawa	27	30	outside	7.50	pale brown	cylindrical	9	2	smooth
951	LC469387	*Ochroconis* sp.	–	Nanjyo	Okinawa	26	30	outside	7.54	pale brown	cylindrical	7	2	smooth

Sampling sites and characteristics of conidia were shown.
Two samples of NH61 and NH512 were collected at 2 sites separated aboout 1 km in Akiyoshidai.

TABLE 2. The growth of *Ochroconis* strains collected from soils of the limestone area cultured on the various media

Sample number	Species	Group	Locality	1/4 PDA		pH 9.7		0.1%SO		0.1%AE		DG18		50°C[1)]	
				colony number	size	colony number	size	colony number	size	colony number	size	colony number	size	colony number	
754	*O. humicola*		Nanjyo	81	+	56	+	58	+	10	+	55	++	25	
2012	*O. humicola*		Ishigaki Is.	69	++	61	+	61	+	8	+	64	++	35	
552	*O. constricta*		Gyokusendo	99	++	70	+	90	+	42	+	57	+	13	
1151	*O. anellii*		Dorogawa	54	+	49	+	50	-	0	-	0	+	18	
33	*O. bacilliformis*		Mt. Ibuki	91	+	42	+	49	-	0	-	0	++	9	
269	*O. cordanae*		Ohtakido	88	+	84	+	77	+	46	+	80	++	16	
232	*O. cordanae*		Mankido	92	+	62	+	40	-	0	+	64	-	0	
1771	*O. cordanae*		Ikurado	82	++	85	+	80	+	72	+	39	+	21	
664	*S. excentricum*		Nyukawa	88	-	0	+	65	-	0	-	0	-	0	
121	*S. excentricum*		Dorogawa	75	-	0	+	34	-	0	-	0	-	0	
2904	*O. globalis*		Ryusendo	59	++	60	+	42	-	0	-	0	+	43	
431	*O. lascauxensis*		Fudokutsu	56	++	64	+	58	-	0	-	0	++	62	
165	*O. macrozamiae*		Mt. Fujiwara	76	+	74	+	77	-	0	+	64	-	0	
222	*O. macrozamiae*		Aguchi	56	-	0	-	0	-	0	-	0	-	0	
1092	*O. macrozamiae*		Dorogawa	54	+	39	+	5	-	0	-	0	-	0	
673	*O. robusta*		Nyukawa	71	+	39	+	37	-	0	-	0	+	1	
236	*O. verruculosa*		Ishigaki Is.	67	+	56	+	67	+	44	+	57	++	17	
280	*Ochroconis* sp.	P	Miyamado	55	++	48	+	52	+	49	-	0	++	48	
14	*Ochroconis* sp.	P	Mt. Ibuki	70	++	71	+	78	-	0	-	0	++	76	
1682	*Ochroconis* sp.	P	Sekigahara	68	+	71	+	69	-	0	+	65	+	48	
185	*Ochroconis* sp.	P	Ikurado	76	+	30	+	34	-	0	+	75	-	0	
61	*Ochroconis* sp.	P	Akiyoshidai	61	+	52	+	54	+	43	-	0	+	58	
512	*Ochroconis* sp.	P	Akiyoshidai	86	+	88	+	90	+	82	-	0	+	80	
685	*Ochroconis* sp.	P	Hiraodai	98	+	102	+	74	-	0	+	88	+	64	
739	*Ochroconis* sp.	P	Mt. Katsuu	94	+	81	+	66	+	12	-	0	++	18	
2902	*Ochroconis* sp.	–	Miyamado	60	+	54	-	0	-	0	-	0	-	0	
41	*Ochroconis* sp.	–	Nippara	98	+	76	+	100	-	0	-	0	-	0	
256	*Ochroconis* sp.	–	Ohtakido	78	+	82	+	62	+	29	+	68	+	44	
273	*Ochroconis* sp.	–	Ohtakido	98	-	0	+	70	+	63	-	0	-	0	
81	*Ochroconis* sp.	–	Shitsushi	82	-	0	+	56	-	0	-	0	-	0	
39	*Ochroconis* sp.	–	Mt. Ibuki	78	-	0	-	0	-	0	-	0	-	0	
136	*Ochroconis* sp.	–	My. Ibuki	50	+	51	+	38	+	38	-	0	+	36	
156	*Ochroconis* sp.	–	Sekigahara	76	+	72	-	0	-	0	-	0	-	0	
152	*Ochroconis* sp.	–	Sekigahara	66	-	0	+	67	-	0	-	0	-	0	
169	*Ochroconis* sp.	–	Mt. Fujiwara	54	+	16	-	0	-	0	-	0	-	0	
230	*Ochroconis* sp.	–	Mankido	57	+	50	-	0	-	0	-	0	-	0	
2211	*Ochroconis* sp.	–	Mankido	68	+	64	+	37	-	0	-	0	+	8	
1751	*Ochroconis* sp.	–	Ikurado	62	+	60	+	57	-	0	-	0	+	48	
4262	*Ochroconis* sp.	–	Fudokutsu	87	-	0	+	56	-	0	-	0	+	23	
52	*Ochroconis* sp.	–	Akiyoshidai	74	++	74	+	77	-	0	+	62	+	5	
721	*Ochroconis* sp.	–	Oku	69	++	55	+	43	-	0	-	0	-	0	
951	*Ochroconis* sp.	–	Nanjyo	87	++	52	+	42	-	0	+	76	+	8	

The count and the size of fungal colony on various culture condition were compared with that on 1/4 PDA medium.
The colony size was compared with that on 1/4 PDA, and assessed as follows: ++, same; +, smaller; -, undetectable.
[1)] Suspension of conidia was incubated at 50°C for 10 min.

DISCUSSION

A unique flora of higher plants and ferns, bryophytes, and lichens is said to be distributed in the limestone areas, although the limestone areas in Japan were covered with grasses, conifer, and deciduous trees (Shimizu, 1963). The alkaline soil seems to affect the fungal flora as well as plant flora. The average pH of soil in outdoor environments, including wild forests, was usually acidic, pH 5.24-5.77 (Hamada and Abe, 2018), but strains of *Ochroconis* isolated in this study were collected in soil from limestone with an average pH of 7.57 (Table 1). The *Ochroconis* strains were often found in the limestone areas as well as in city parks (pH 6.89 on average), and most fungi were able to grow on alkaline media and on the media with an added soap component. Thus, compared to general wild environments, the limestone areas seem to provide better conditions for many strains of *Ochroconis*.

However, conditions that are too alkaline, such as those inside limestone caves, repress fungal growth (Pitt and Hocking, 2009). Moreover, organic substances that can be used as nutrients are less abundant inside the caves rather than outside; thus, the number of fungi was lower in samples collected from inside the cave. However, the distribution of *Ochroconis* seems to be similar, regardless of the region of sample collection; thus, we supposed that the genus *Ochroconis* has generally become adapted to alkaline environments.

The microclimate of the limestone areas was thought to be more variable than that of city parks, because the limestone areas are variable geographically and covered with various plants. Moreover, the environments in city parks change easily with regard to temperature and humidity, and hence it is more difficult for fungi to survive in city parks than in limestone. Therefore, *Ochroconis* strains with various genetic and physiological characteristics were able to grow in limestones, although their pH is generally more alkaline than in city parks. Twenty-eight species were identified in 42 strains from limestones, compared with 8 species in 32 strains from city parks, or 3 species in 24 strains from indoors, as reported previously (Hamada and Abe, 2018). The diversity of *Ochroconis* was very high in limestones, although *Ochroconis* species are difficult to find in other areas than limestones. Thus, we assumed that many *Ochroconis* species originally distributed in limestones spread to city parks and indoor environments.

In a previous study (Hamada and Abe, 2018), three (*O. constricta,* Group P, and *O. humicola*) out of eight species of *Ochroconis* from city parks grew on media containing non-ion surfactants, and the ability of *O. humicola* to use surfactants was higher than that of other *Ochroconis* species. In this study, 8 out of 28 species collected in the limestone areas grew using non-ion surfactants as nutrients. Phylogenetic analysis showed that the characteristic of using non-ion surfactants was found randomly in many clusters. Thus, the ability to use non-ion surfactants as nutrients appears to be distributed throughout the genus of *Ochroconis*. The species using surfactants could be candidates for immigration to indoor environments, although only *O. humicola* was found predominantly indoors.

Although other factors besides the ability to use non-ion surfactants were thought to affect propagation in the indoor environments, the ability to grow under alkaline conditions affected immigration in the previous study (Hamada and Abe, 2018). Moreover, heat tolerance and drought tolerance were found to be linked to the utilization of non-ion surfactants in this study (Table 2). *Ochroconis humicola* possesses all of these abilities, unlike other *Ochroconis* species.

In this study, we obtained many strains of *Ochroconis* from the limestone areas in the Honshu islands (farther north than 30N) as well as from urban city parks, as described previously (Hamada and Abe, 2018) but we could not find any *O. humicola* outside of these regions. However, in the southern islands, located farther south than 30N, *O. humicola* was found for the first time in limestones in this study, as well as outdoors in two city parks, as described previously (Hamada and Abe, 2018). Namely, *O. humicola* is suggested to have been distributed originally in alkaline soils in sub-tropical regions.

Ochroconis humicola from the limestone areas spread to city parks and indoor environments, and then to indoor environments all over the world, including Asia, Europe, and USA. Subsequently, *O. humicola* became predominant in the indoor environments, with the characteristic of using detergents more effectively than other fungi. However, *O. humicola* from outdoor environments has not spread to the northern regions.

Ochroconis strains, which were not heat-tolerant or drought-tolerant, were predominantly found in the limestone areas, but not in city parks. This result suggests that the environmental conditions of limestones are generally milder and moister than those in city parks. In this study, the Group P strains of *Ochroconis* were also found in limestones, although the strains were found more commonly in the soils from city parks. Group P strains may have occurred originally in the limestone areas and then immigrated to the urban city parks with the dust of cement and seem to have adapted to hot and dry conditions.

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