

Supplementary Figure 1. **Multiple alignment of *BAD1* gene of *B. dermatitidis* and *B. gilchristii*.** The sequences of *BAD1* gene from *B. dermatitidis* (*Bd*) and *B. gilchristii* (*Bg*) were aligned using MUSCLE alignment program in Geneious. The gray area shows the consensus sequences between *Bd* and *Bg* while the color area shows the non-consensus sequences, and the line indicates deletion of nucleotide sequences. The *Bd* probe V2560 is stretched because the alignment with *Bg* sequences had insertions at the assigned region.



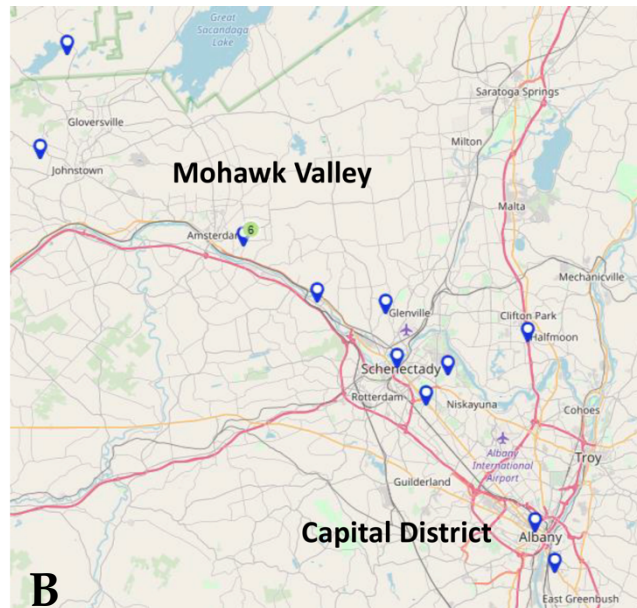
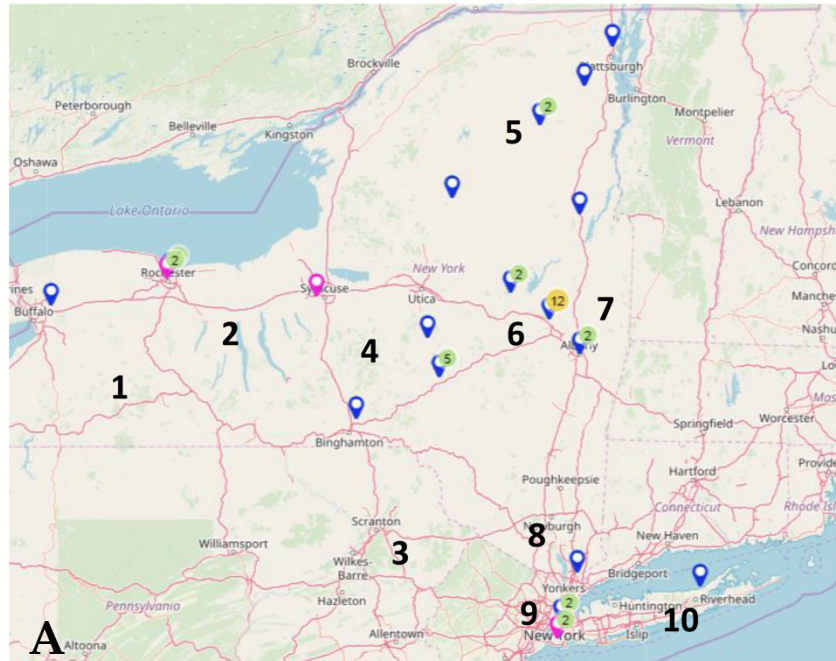
Bg



Bd

1. Western New York
2. Finger Lakes
3. Southern Tier
4. Central New York
5. North Country
6. Mohawk Valley
7. Capital District
8. Hudson Valley
9. New York City
10. Long Island

Map made using Mapline
(<https://mapline.com/>)



Supplementary Figure 2. **Geographic distribution of blastomycosis cases in New York from 2005 to 2019.** (A) Patient's residence (Zip Code) was utilized to map cases of blastomycosis due to *B. dermatitidis* (*Bd*) and *B. gilchristii* (*Bg*) in New York. (B) Clustering of cases in Mohawk Valley and Capital District, and (C) Finger Lakes, are shown.

Supplementary Table 1. Details of duplex real-time PCR assay of Blastomycosis cases from New York (2005-2019)

Case No.	Primary Sample Collection Date	Source <i>Blastomyces</i> recovered in culture	DNA Concentration (ng/ μ L)	Single plex real-time PCR (<i>Blastomyces</i> spp.) Ct (FAM)	Duplex real-time PCR		Final ID
					<i>Bd</i> Mean Ct (Cy5)	<i>Bg</i> Mean Ct (FAM)	
Case 1	7/14/2005	Bone	6.48	22	22.03	Undet	<i>B. dermatitidis</i>
Case 2	2/23/2006	BAL	4.98	20.7	20.71	Undet	<i>B. dermatitidis</i>
Case 3	8/10/2006	Tissue	3.92	20.7	22.57	Undet	<i>B. dermatitidis</i>
Case 4	1/17/2008	Tissue	5.08	22.5	22.57	Undet	<i>B. dermatitidis</i>
Case 4	1/17/2008	Tissue	9.98	21.5	20.51	Undet	<i>B. dermatitidis</i>
Case 4	1/17/2008	Tissue	7.22	21.2	20.46	Undet	<i>B. dermatitidis</i>
Case 5	4/23/2008	BAL	7.32	20.19	Undet	20.67	<i>B. gilchristii</i>
Case 6	8/17/2009	Tissue	6.02	22.01	21.31	Undet	<i>B. dermatitidis</i>
Case 7	11/18/2009	Tissue	1.61	Culture**	24.17	Undet	<i>B. dermatitidis</i>
Case 8	2/10/2010	LUNG	2.58	22.29	Undet	22.75	<i>B. gilchristii</i>
Case 9	3/1/2012	BAL	31.8	23.33	22.43	Undet	<i>B. dermatitidis</i>
Case 10	9/26/2012	Lung	47	20.97	19.47	Undet	<i>B. dermatitidis</i>
Case 11	6/6/2013	Aspirate	23.8	20.94	18.57	Undet	<i>B. dermatitidis</i>
Case 12	10/25/2013	Bone	3.92	18.8	23.58	Undet	<i>B. dermatitidis</i>
Case 13	11/19/2013	BAL	5.56	20.54	22.12	Undet	<i>B. dermatitidis</i>
Case 14	2/11/2014	Skin lesion fluid*	21.2	28.25	20.23	Undet	<i>B. dermatitidis</i>
Case 15	2/17/2014	Sputum	9.74	20.52	22.47	Undet	<i>B. dermatitidis</i>
Case 15	2/18/2014	BAL	0.676	24.34	21.46	Undet	<i>B. dermatitidis</i>
Case 16	5/29/2014	BAL	33	27.63	20.73	Undet	<i>B. dermatitidis</i>
Case 16	6/12/2014	BAL	41.8	21.73	18.99	Undet	<i>B. dermatitidis</i>
Case 17	8/8/2014	Brain	0.766	28.99	27.95	Undet	<i>B. dermatitidis</i>
Case 18	9/29/2015	Cyst	1.21	26.94	24.04	Undet	<i>B. dermatitidis</i>
Case 19	10/20/2016	Hand	3.7	27.7	20.68	Undet	<i>B. dermatitidis</i>
Case 20	1/13/2017	Lung	27	24.36	20.14	Undet	<i>B. dermatitidis</i>
Case 21	1/16/2017	BAL	47.2	26.33	Undet	19.26	<i>B. gilchristii</i>
Case 22	3/1/2017	Lung	7.36	26.79	23.74	Undet	<i>B. dermatitidis</i>
Case 23	5/2/2017	Sputum	12.1	21.73	21.07	Undet	<i>B. dermatitidis</i>
Case 24	5/17/2017	BAL	22	22.01	20.37	Undet	<i>B. dermatitidis</i>
Case 25	6/28/2017	Tissue	2.76	23.22	23.06	Undet	<i>B. dermatitidis</i>
Case 26	10/26/2017	BAL	2.5	21.19	23.49	Undet	<i>B. dermatitidis</i>
Case 26	10/26/2017	BAL	0.866	21.34	24.37	Undet	<i>B. dermatitidis</i>
Case 26	10/27/2017	Sputum	5.38	32.47	36.56	Undet	<i>B. dermatitidis</i>
Case 26	10/27/2017	Sputum	40.8	29.71	32.02	Undet	<i>B. dermatitidis</i>
Case 26	10/27/2017	Sputum	26	26.8	29.58	Undet	<i>B. dermatitidis</i>
Case 27	10/3/2018	Bone - Left Tibia	4.48	19.11	21.54	Undet	<i>B. dermatitidis</i>
Case 28	2/13/2018	Bone (Tibia)	1.37	20.05	22.71	Undet	<i>B. dermatitidis</i>
Case 29	3/6/2018	Tissue block - Left nasal Mass*	1.46	37.4	39.63	Undet	<i>B. dermatitidis</i>

Case 29	3/6/2018	Tissue block - Left nasal Mass*	2.3	33.29	37.24	Undet	<i>B. dermatitidis</i>
Case 30	2/28/2018	Tissue - Left Thigh	0.298	20.47	23.58	Undet	<i>B. dermatitidis</i>
Case 31	8/5/2018	BAL	0.658	22.63	27.74	Undet	<i>B. dermatitidis</i>
Case 32	7/2/2018	BAL	15	22.89	27.36	Undet	<i>B. dermatitidis</i>
Case 33	9/27/2018	Tissue block*	1.71	36.02	40.29	Undet	<i>B. dermatitidis</i>
Case 34	10/2/2018	Bone (Femur)	9.86	19.09	22.77	Undet	<i>B. dermatitidis</i>
Case 35	12/17/2018	BAL	1.72	19.72	Undet	22.49	<i>B. gilchristii</i>
Case 36	1/20/2019	Tissue	0.434	20.87	24.81	Undet	<i>B. dermatitidis</i>
Case 37	4/25/2019	BAL	0.48	21.01	24.54	Undet	<i>B. dermatitidis</i>
Case 38	5/5/2019	BAL*	5.6	23.54	27.13	Undet	<i>B. dermatitidis</i>
Case 39	5/9/2019	BAL	0.336	23.8	24.52	Undet	<i>B. dermatitidis</i>
Case 40	5/23/2019	Wound fluid	1.64	20.16	23.26	Undet	<i>B. dermatitidis</i>
Case 41	7/2/2019	BAL	11.5	18.74	23.06	Undet	<i>B. dermatitidis</i>
Case 42	7/19/2019	BAL	23.5	19.45	24.28	Undet	<i>B. dermatitidis</i>
Case 43	10/22/2019	BAL	10.4	18.88	Undet	17.73	<i>B. gilchristii</i>
Case 43	10/22/2019	BAL	13.6	18.74	Undet	17.73	<i>B. gilchristii</i>

*Primary samples; ** Culture was confirmed by converting to the yeast form

Supplementary Table 2. Specificity study of duplex real-time PCR assay

Fungal ID	Source	Isolate DNA (ng/ μ L)	Duplex rt-PCR Result
<u>MOLDS</u>			
<i>Alternaria alternata</i>	Nasal cavity	108.3	Negative
<i>Aspergillus flavus</i>	Sputum	60.3	Negative
<i>A. fumigatus</i>	Sputum	18.5	Negative
<i>A. nidulans</i>	BAL	54.2	Negative
<i>A. niger</i>	Nasal cavity	147.1	Negative
<i>A. versicolor</i>	Lung	123.4	Negative
<i>Blastobotrys adeninivorans</i>	CSF	255.8	Negative
<i>Blastomyces persicus</i> (ATCC 56214)	Human (Mozambique)	101.5	Negative
<i>B. emzantsi</i> (ATCC 56216)	Human (South Africa)	159.2	Negative
<i>Cladosporium halotolerans</i>	Nail	92.8	Negative
<i>C. ramotenellum</i>	Sputum	2.2	Negative
<i>Chaetomium globosum</i>	Nail	90.1	Negative
<i>Chrysosporium queenslandicum</i>	BAL	21.2	Negative
<i>C. queenslandicum</i>	No source	50.1	Negative
<i>Coccidioides immitis</i>	BAL	6.9	Negative
<i>C. posadasii</i>	BAL	18.2	Negative
<i>C. posadasii</i>	Sputum	120	Negative
<i>Epidermophyton floccosum</i>	Nail	34	Negative
<i>Exophiala dermatitidis</i>	BAL	110.8	Negative

<i>E. lecanii-corni</i>	Nail	102.4	Negative
<i>Fusarium oxysporum</i>	Nail	152.4	Negative
<i>Geomyces pannorum</i>	BAL	280.4	Negative
<i>Glomerella lagenaria</i>	BAL	3.6	Negative
<i>Histoplasma capsulatum</i>	Blood	110.7	Negative
<i>H. capsulatum</i>	Lung	17.7	Negative
<i>Hyphopichia burtonii</i>	Mouth	302.5	Negative
<i>Lichtheimia corymbifera</i>	BAL	10.5	Negative
<i>Malbranchea circinata</i>	Nail	19.8	Negative
<i>Microsporium gypseum</i>	Nail	318.7	Negative
<i>M. canis</i>	Scalp	117.4	Negative
<i>Phoma herbarum</i>	Nail	91.2	Negative
<i>Phlebia fuscoatra</i>	Sputum	28.9	Negative
<i>Rhizopus oryzae</i>	Urine	116.4	Negative
<i>Scedosporium boydii</i>	Nail	53.3	Negative
<i>Sporothrix schenckii</i>	Finger	72.8	Negative
<i>Trametes versicolor</i>	BAL	43.2	Negative
<i>T. versicolor</i>	Eye	17.8	Negative
<i>Trichosporon asahii</i>	Urine	132.4	Negative
<i>Trichophyton mentagrophytes</i>	Nail	62.6	Negative
<i>T. rubrum</i>	Nail	9.3	Negative
<i>T. violaceum</i>	Scalp	33.9	Negative
YEASTS			
<i>Candida albicans</i>	BAL	252.4	Negative
<i>C. auris</i>	Blood	180.8	Negative
<i>C. auris</i>	Blood	108.6	Negative
<i>C. auris</i>	Blood	173.8	Negative
<i>C. duobushaemulonii</i>	Nail	112.6	Negative
<i>C. glabrata</i>	Blood	127	Negative
<i>C. haemulonii</i>	Tissue	55.7	Negative
<i>C. haemulonii</i>	Bone	149	Negative
<i>C. krusei</i>	Blood	3	Negative
<i>C. lusitaniae</i>	Blood	95.8	Negative
<i>C. orthopsilosis</i>	Abdominal fluid	52.6	Negative
<i>C. parapsilosis</i>	Blood	78.5	Negative
<i>C. tropicalis</i>	Sputum	101.5	Negative
<i>Cryptococcus albidus</i>	Nail	7.9	Negative
<i>C. diffluens</i>	No source	6.2	Negative
<i>C. gattii</i> (VG1)	CSF	54.7	Negative
<i>C. liquefaciens</i>	Right Posterior Ankle	82.7	Negative
<i>Debaryomyces hansenii</i>	Foot	48	Negative
<i>Pichia norvegensis</i>	No source	194.8	Negative
<i>Saccharomyces cerevisiae</i>	Vaginal swab	100.8	Negative
Pos Ctrl Bg (MB97)	N/A	10.0	Positive
Pos Ctrl Bd (M808)	N/A	15.5	Positive

Supplementary Table 3 A. Inter-assay reproducibility of duplex real-time PCR assay

<i>Blastomyces</i> spp.	Strain No.	<i>B. dermatitidis</i> (Cy5)				<i>B. gilchristii</i> (FAM)				±SD	%CV
		Ct 1	Ct 2	Ct 3	Mean Ct	Ct 1	Ct 2	Ct 3	Mean Ct		

<i>B. gilchristii</i>	MB95	Undet	Undet	Undet	N/A	22.9	22.47	21.35	22.24	0.8	3.59
	MB96	Undet	Undet	Undet	N/A	24.21	23.68	23.2	23.7	0.51	2.15
	MB97	Undet	Undet	Undet	N/A	23.78	23.24	22.37	23.13	0.71	3.07
<i>B. dermatitidis</i>	MB98	24.88	25.63	25.09	25.2	Undet	Undet	Undet	N/A	0.39	1.55
	MB99	24.18	24.97	24.24	24.46	Undet	Undet	Undet	N/A	0.44	1.8
	MB100	22.68	23.42	22.49	22.86	Undet	Undet	Undet	N/A	0.49	2.14

Supplementary Table 3 B. Intra-assay reproducibility of duplex real-time PCR assay

<i>Blastomyces</i> spp	Strain No.	<i>B. dermatitidis</i> (Cy5)				<i>B. gilchristii</i> (FAM)				±SD	%CV
		Ct 1	Ct 2	Ct 3	Mean Ct	Ct 1	Ct 2	Ct 3	Mean Ct		
<i>B. gilchristii</i>	MB95	Undet	Undet	Undet	N/A	22.99	22.85	22.88	22.91	0.0737	0.322
	MB96	Undet	Undet	Undet	N/A	24.24	24.15	24.23	24.21	0.0493	0.204
	MB97	Undet	Undet	Undet	N/A	23.77	23.79	23.78	23.78	0.01	0.0421
<i>B. dermatitidis</i>	MB98	24.91	24.87	24.87	24.88	Undet	Undet	Undet	N/A	0.0231	0.0928
	MB99	24.03	24.15	24.36	24.18	Undet	Undet	Undet	N/A	0.167	0.691
	MB100	22.81	22.69	22.55	22.68	Undet	Undet	Undet	N/A	0.13	0.573