The Journal of Veterinary Medical Science

NOTE

Bacteriology

Validation of the usefulness of 26S rDNA D1/D2, internal transcribed spacer, and intergenic spacer 1 for molecular epidemiological analysis of *Macrorhabdus ornithogaster*

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J. Vet. Med. Sci.
84(2): 244–250, 2022
doi: 10.1292/jvms.21-0576

Received: 27 October 2021
Accepted: 12 December 2021
Advanced Epub:
23 December 2021

ABSTRACT. *Macrorhabdus ornithogaster* (MO) is an infectious fungus that causes gastric damage in birds. In this study, we established nested and seminested polymerase chain reaction (PCR) methods that specifically amplify the domain D1/D2 region (D1/D2) of 26S ribosomal DNA (rDNA), internal transcribed spacer (ITS) of rDNA, and intergenic spacer (IGS) 1 region from avian feces. Phylogenetic analysis of MO collected from Japanese pet birds showed little genetic variation; analysis based on these regions did not distinguish between host species order, differences in MO shape, or host gastrointestinal symptoms. These regions were found to be unsuitable for molecular epidemiological studies of MO and further investigation into other genetic regions is required.

KEY WORDS: 26S rDNA D1/D2, genotyping, intergenic space 1, internal transcribed spacer, *Macrorhabdus ornithogaster*

Macrorhabdus ornithogaster (MO) is a long, straight, narrow, rod-shaped fungus (2–3 µm × 20–80 µm) that grows solely at the junction of the proventriculus and ventriculus in birds [2, 15]. MO is distributed worldwide and exhibits a wide host range, which includes psittacine and passerine birds, poultry, and several other species [8]. Some MO-infected birds develop macrorhabdosis, which causes acute gastric disturbances or slow weight loss (termed as "going light"), leading to death in severe cases. As such, MO-infected birds are at serious risk [7]. Fortunately, the genomic information on MO has gradually increased over recent years. Because MO was once considered a bacterium [15], it was referred to as a "megabacterium" or collectively as "megabacteria". However, Tomaszewski *et al.* [14] identified the organism as an anamorphic ascomycetous yeast by analyzing ribosomal DNA (rDNA), particularly the 18S rDNA and the domain D1/D2 region (D1/D2) of 26S rDNA. Phylogenetic analysis of the internal transcribed spacer (ITS) of rDNA has also been reported in studies conducted in Iran and Germany [1, 9]. Abdi-Hachesoo *et al.* [1] found that MO can be divided into A and B genotypic groups based on genotyping of the ITS region, while the hosts of each group can be divided into Passeriformes and Psittaciformes. This trend was also observed in a survey by Püstow *et al.* [9]. However, it is not clear how the MO genotype is related to the development of macrorhabdosis.

The purpose of this study was to test whether the IGS1 region (which is useful for phylogenetic analysis of other fungi) and the D1/D2 and ITS regions (for which MO sequence information has been gathered) are useful for molecular epidemiological analysis of MO. However, the samples for phylogenetic analysis of MO are limited to the gastric mucosa and its culture [1, 9, 14], which are difficult to obtain. Therefore, we first established a nested polymerase chain reaction (PCR) method to specifically amplify these regions in avian feces. Next, we conducted a phylogenetic analysis of MO from Japanese pet birds to determine whether genotyping based on these regions can distinguish between MO morphology and reveal the presence/absence of gastrointestinal symptoms in the host.

Stool samples were collected individually from pet birds that visited the "Little Bird and Small Animal Hospital LITTLE

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BIRD" (Tokyo, Japan) over the course of one year and the presence of MO was detected via direct wet mount microscopy. For the MO culture, 75 bird droppings were collected and stored at 4°C for 1–7 days. According to the method outlined in a previous study [4], MO-containing fecal samples were cultured in 24-well plates comprising the Eagle's basal medium supplemented with 20% heat-inactivated fetal bovine serum, 5% sucrose, and antibiotics with pH adjusted to 3.5. Culture tubes with double-layered caps (4 ml culture tube PP, WATSON, Tokyo, Japan) were used to prevent contamination and placed in a simple anaerobic culture system (Anaero pack, Mitsubishi Gas Chemical Co., Tokyo, Japan) at 42°C. Tubes contaminated with other fungi were discarded. This protocol was repeated weekly for 1.5 years. Cockatiel and budgerigar-derived fecal samples (sample no. M34 and M50, respectively) were successfully subcultured using this method, and the culture strains were named SuMO1 and SuMO2, respectively (Fig. 1, Table 1).

In addition to the culture experiments, 17 bird droppings were collected for MO genotyping and frozen at −80°C for eventual DNA extraction. The profiles of 17 birds (from which samples were successfully cultured and collected for genotyping) were investigated for classification, age, and symptoms by using the medical records and included two orders, four families, six genes, and six species. Of the 17 birds, 13 were less than one year old. A total of eight birds had gastrointestinal symptoms: three budgerigars (*Melopsittacus undulatus*), one parrotlet (*Forpus coelestis*), three cockatiel (*Nymphicus hollandicus*) juveniles, and one zebra finch (*Taeniopygia guttata*). No gastrointestinal symptoms were observed in any of the other birds.

The cultured MO or naturally thawed feces were centrifuged and pelleted. To lyse the robust cell wall of MO, 5 µl of zymolyase

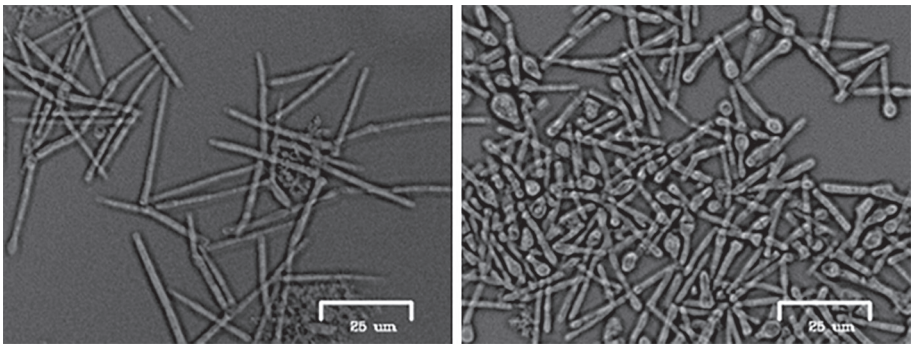

Fig. 1. Microscopic morphology of cultured *Macrorhabdus ornithogaster* (scale bar=25 µm). The left panel shows a budgerigar-derived strain (SuMO2) and the right panel shows a cockatiel-derived strain (SuMO1). SuMO2 had an elongated rod-like morphology, while SuMO1 was thicker and shorter, often with a ball-like tip (similar to a drumstick).

Table 1. The bird information used in this study

Order	Species	Scientific names	Bird no.	Sample no.	Strain names	DDBJ accession no.			Reason for encounter	Age
						ITS	D1D2	IGS1		
Psittaciformes	Cockatiel	*Nymphicus hollandicus*	1	M34	SuMO1	LC633765	LC633783	LC633289	MC	1M
				M35		LC633766	LC633784	LC633801		
			2	M55		LC633767	LC633785	LC633802	MC	3M
			3	M56		LC633768	LC633786	LC633803	MC	2M
			4	M67		LC633769	LC633787	LC633804	●Vomit, ●Anorexia, ●Going light	1M
			5	M68		LC633770	LC633788	LC633805	●Emaciation	2M
			6	M69		LC633771	LC633789	LC633806	●Emaciation	2M
	Pacific parrotlet	*Forpus coelestis*	7	M64		LC633772	LC633790	LC633807	●Anorexia, ●Emaciation	1M
			8	M71		LC633773	LC633791	LC633808	MC	1M
	Budgerigar	*Melopsittacus undulatus*	9	M50	SuMO2	LC633774	LC633792	LC633809	●Emaciation	2Y
			10	M14		LC633775	LC633793	LC633810	●Melena	8M
			11	M23		LC633776	LC633794	LC633811	MC	2M
			12	M8		LC633777	LC633795	LC633812	●Anorexia	3Y
			13	M9		LC633778	LC633796	LC633813	MC	1Y
	Rosy-faced Lovebird	*Agapornis roseicollis*	14	M15		LC633779	LC633797	LC633814	MC	1M
Passeriformes	Java sparrow	*Lonchura oryzivora*	15	M59		LC633780	LC633798	LC633815	MC	Unknown
	Zebra finch	*Taeniopygia guttata*	16	M19		LC633781	LC633799	LC633816	●Anorexia, ●Diarrhea	>2Y
			17	M60		LC633782	LC633800	LC633817	Trauma	3M

D1D2, domain D1/D2; ITS, internal transcribed spacer; IGS1, intergenic space 1; MC, medical checkup; ●, symptom of gastrointestinal disorders; M, month; Y, year.

(Longlife Zymolyase, G-Biosciences, St. Louis, MO, USA) was added to approximately 50 µl of the pellet and incubated at 37°C for 30 min. The lysed pellets were ground with a masher (Nippi Inc., Tokyo, Japan), and DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol (the melting temperature was 90°C for 5 min). The DNA extract was stored at −30°C.

First, specific primers were designed for amplification of the ITS region and D1/D2 region sequences of MO, according to the MO sequence in the database (GenBank accession no. AF350243.1). As the DNA extracted from fecal samples contained other fungal DNA, MO-specific primers were designed for seminested PCR (MO18SF2 and MO26SR3, 26SR2). The specificity of the primers was confirmed using the basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast/). Panfungal primers (ITS3) were also used to amplify the D1/D2 region (Fig. 2A, Table 2) [16].

In the nested PCR for ITS and D1/D2 region amplification, primer set 1 (MO18SF2 and MO26SR3, 1,385 bp) was used for the first PCR run, and primer sets 2 (MO18SF2 and 26SR2, 699 bp) and 3 (ITS3 and MO26SR3, 1,070 bp) were used for the second run to amplify the ITS and D1/D2 regions, respectively (Fig. 2A, Table 2).

SeqAmp DNA polymerase (Takara Bio Inc., Kusatsu, Japan) was used for PCR amplification according to the manufacturer's protocol; initial denaturation at 94°C for 1 min, 30 cycles of denaturation at 98°C for 10 sec, annealing at 55°C or 60°C for 15 sec, and extension at 68°C for 30 sec/kb.

The PCR products were confirmed using agarose gel electrophoresis for band expression. In the first PCR run, no bands were observed for any of the primer sets used. However, amplification was observed in the second run for both D1/D2 and ITS.

The PCR product was purified using a MonoFas DNA Purification Kit I (ANIMOS Inc., Tokyo, Japan) and their nucleotide sequences were determined via the Fasmac DNA sequencing service (Fasmac, Atsugi, Japan) using the primers 18SF2 and 26SR3. The obtained sequences were completely or highly homologous to the MO sequence in the GenBank database (Table 3), thereby confirming that the ITS and D1/D2 region sequences can be obtained directly from avian fecal samples using the method developed in this study.

Next, we established a method to obtain the sequence of the IGS1 region, which is commonly used in the phylogenetic analysis of other pathogenic fungi. As the IGS1 sequence of MO is not registered in any database, the unknown rDNA sequence (including the IGS region) was determined using next generation sequencing (NGS). An MO-specific forward primer (MO26SF2) was designed based on the 3′ end of the 26S rDNA, which was combined with a reverse primer (26SR2) on the 5′ end. (primer set 4) (Fig.

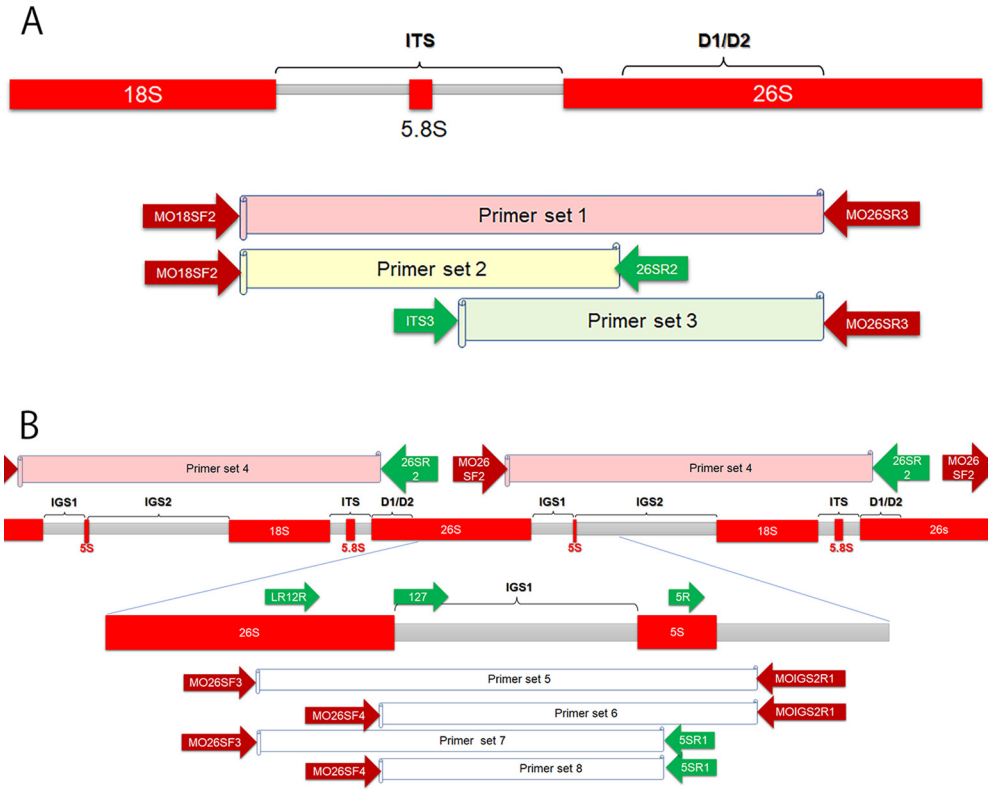

Fig. 2. In conventional PCR, to amplify the D1D2 and internal transcribed spacer (ITS) regions, primer set 1 (MO18SF2 and MO26SR3), primer set 2 (MO18SF2 and 26SR2), and primer set 3 (ITS3 and MO26SR3) were used. In nested PCR, primer set 1 was used for the first run, and primer sets 2 (ITS) and 3 (D1D2) were used for the second run (**A**). Primer set 4 (MO26SF2 and 26SR2) was used in conventional PCR to amplify the *Macrorhabdus ornithogaster* (MO) rDNA sequence containing the unknown intergenic spacer (IGS) region. In nested PCR, to amplify the IGS1 region, primer set 5 (MO26SF3 and MOIGS2R1) and primer set 7 (MO26SF3 and 5SR1) were used for the first run, and primer set 6 (MO26SF3 and MOIGSR1) and primer set 8 (MO26SF3 and 5SR1) were used for the second run (**B**).

Table 2. Primers used in this study

Primer names	Forward or Reverse	Specificity	Sequences
MO18SF2	Forward	Specific[a]	5′- AGGAGGCGACTCCATTCTATAGTG -3′
MO 26SR1	Reverse	Specific [a]	5′- CTCTATGGCATCCTTTTCCAAGG -3′
26SR2	Reverse	Semi Specific [b]	5′- TGAGCTTTTACCACTTCACTCGC -3′
MO26SR3	Reverse	Specific [a]	5′- CCTTCTAGATCGGTCGATTATGC -3′
ITS1	Forward	Panfungal [c]	5′- TCCGTAGGTGAACCTGCGG -3′
ITS3	Reverse	Panfungal [c]	5′- GCATCGATGAAGAACGCAGC -3′
ITS4	Reverse	Panfungal [c]	5′- TCCTCCGCTTATTGATATGC -3′
NL1	Forward	Panfungal [c]	5′- GCATATCAATAAGCGGAGGAAAAG -3′
NL4	Reverse	Panfungal [c]	5′- GGTCCGTGTTTCAAGACGG -3′
MO26SF2	Forward	Specific [a]	5′- T GATGGCGCTTTAGCGTGATACCTATAC -3′
MO26SF4	Forward	Specific [a]	5′- GCAGTTGTTTGAAAAAGCATTGCTGCG -3′
MO26SF3	Forward	Specific [a]	5′- CGCAGAACCATATTAGATTAGCGATAG -3′
5SR1	Reverse	Nonspecific A [d]	5′- CCGTCCGATCAACTGTAGTTAAGC -3′
MOIGS2R1	Reverse	Specific	5′- CTTACTCTTATCCATTTAATCCTCAC-3′
LR12R	Forward	Nonspecific [d]	5′- CTGAACGCCTCTAAGTCAGAA -3′
5SR	Reverse	Nonspecific B [e]	5′- TCCGATCAACTGTAGTTAAG -3′

[a] *Macrorhabdus ornithogaster* (MO) specific primer, [b] Specific primer for MO and a few fungi, [c] Panfungal primer, [d] Non-specific primer designed for MO, [e] Non-specific primers designed for other fungi.

Table 3. Reference sequence obtained from NCBI database

Locus	GenBanK accession no.	Organism	Derived bird species
D1D2	AF3502431	*Macrorhabdus ornithogaster*	*Melopsittacus undulatus*
	KX426594	*Macrorhabdus ornithogaster*	*Melopsittacus undulatus*
	KX426595	*Macrorhabdus ornithogaster*	*Taeniopygia guttata*
	KX426596	*Macrorhabdus ornithogaster*	*Carduelis carduelis*
	KX426597	*Macrorhabdus ornithogaster*	*Carduelis carduelis*
	NR132207	*Saccharomyces cerevisiae*	
	AB217514	*Kluyveromyces nonfermentans*	
ITS	AF350243	*Macrorhabdus ornithogaster*	*Melopsittucus unduratus*
	KX426586	*Macrorhabdus ornithogaster*	*Melopsittucus unduratus*
	KX426587	*Macrorhabdus ornithogaster*	*Nephema splendida*
	KX426591	*Macrorhabdus ornithogaster*	*Taeniopygia guttata*
	KX426592	*Macrorhabdus ornithogaster*	*Serinus canaria*
	KX426593	*Macrorhabdus ornithogaster*	*Carduelis carduelis*
	KX426588	*Macrorhabdus ornithogaster*	*Carduelis carduelis*
	EU649673	*Saccharomyces cerevisiae*	
	AB012264	*Kluyveromyces nonfermentans*	
IGS1	DQ130072	*Saccharomyces cerevisiae*	
	LR738911	*Kluyveromyces marxianus*	

D1D2, domain D1/D2; ITS, internal transcribed spacer; IGS1, intergenic space 1.

2B, Table 2). Using the protocol described previously, PCR was performed with SeqAmp DNA polymerase by using DNA extracted from SuMO1 as the template (Table 1); subsequently, the PCR product was purified using the MonoFas DNA Purification Kit I and used for library preparation for NGS analysis with the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA). NGS was performed using a MiSeq benchtop sequencer (Illumina) and the MiSeq Reagent Kit v3 (150 cycles; Illumina) with 75 paired-end reads. Fastq files created by the MiSeq Reporter (Illumina) were imported into the CLC Genomics Workbench 12.0.3 (CLC bio, Aarhus, Denmark). The obtained reads were trimmed using the quality control tool of the CLC Genomics Workbench with default parameters. The sequences of the obtained contigs were aligned with the primers for IGS1 amplification of other fungi (LR12R, 5SR) [3, 6] and the IGS1 sequence of *Saccharomyces cerevisiae* (GenBank accession no. DQ130072) to estimate the IGS1 region in MO (Fig. 2B, Table 3).

From the sequences revealed by NGS, we designed new primers to amplify the IGS1 region of MO and the specificity of the four newly designed primers (MO26SF4, MO26SF3, MOIGS2R1, and 5SR1) was confirmed by a BLAST search (Table 2).

In nested PCR for IGS1 region amplification, primer set 6 (MO26SF3 and MOIGS2R1, 930 bp) was used in the first PCR run, while primer set 8 (MO26SF3 and 5SR1, 843 bp) was used in the second run. For samples in which no band could be identified in the second PCR, the combination of primer set 5 (MO26SF4 and MOIGSR1, 780 bp) and primer set 7 (MO26SF4 and 5SR1, 693 bp) was used (Fig. 2B, Table 2).

SeqAmp DNA polymerase was used for PCR amplification by following the protocol described previously, after which the PCR product was purified using a MonoFas DNA Purification Kit I. Their nucleotide sequences were determined with Fasmac DNA sequencing by using the primers 26SF3 and 5SR1. The sequence obtained from fecal sample M35 was not registered in any database (Table 3) and was completely homologous to the sequence of sample M34 (SuMO1) cultured from the same bird feces analyzed by NGS, confirming that the sequence of the IGS1 region can be obtained directly from avian fecal samples.

Finally, we performed phylogenetic analysis of the ITS, D1/D2, and IGS1 region sequences from the two cultures and 16 avian fecal

Table 4. Single nucleotide polymorphism in the D1D2 region

Sample no.	DDBJ accession no.	Derived bird species	Polymorphism Sequences (5′-3′)
M14	LC633793	*Melopsittacus undulatus*	AGAAGTGTTATAG
M15	LC633797	*Agapornis roseicollis*	AGAAGTGTTATAG
M19	LC633799	*Taeniopygia guttata*	AGAAGTGTTATAG
M23	LC633794	*Melopsittacus undulatus*	AGAAGTGTTATAG
M34	LC633783	*Nymphicus hollandicus*	AGAAGTGTTATAG
M35	LC633784	*Nymphicus hollandicus*	AGAAGTGTTATAG
M50	LC633792	*Melopsittacus undulatus*	AGAAGTGTTATAG
M55	LC633785	*Nymphicus hollandicus*	AGAAGTGTT**G**TAG
M56	LC633786	*Nymphicus hollandicus*	AGAAGTGTTATAG
M59	LC633798	*Lonchura oryzivora*	AGAAGTGTTATAG
M60	LC633800	*Taeniopygia guttata*	AGAAGTGTTATAG
M64	LC633790	*Forpus coelestis*	AGAAGTGTTATAG
M67	LC633787	*Nymphicus hollandicus*	AGAAGTGTTATAG
M68	LC633788	*Nymphicus hollandicus*	AGAAGTGTTATAG
M69	LC633789	*Nymphicus hollandicus*	AGAAGTGTT**G**TAG
M71	LC633791	*Forpus coelestis*	AGAAGTGTTATAG
M8	LC633795	*Melopsittacus undulatus*	AGAAGTGTTATAG
M9	LC633796	*Melopsittacus undulatus*	AGAAGTGTTATAG
	KX426595	*Taeniopygia guttata*	AGAAGTGTTATAG
	KX426596	*Carduelis carduelis*	AGAAGTGTTAT**G**G
	KX426597	*Carduelis carduelis*	**G**GAAGTGTTATAG
	AF3502431	*Melopsittacus undulatus*	AGAAGTGTTATAG
	KX426594	*Melopsittacus undulatus*	AGAAGTGTTATAG

Bold and underlined font is the single nucleotide polymorphism.

samples. The obtained sequences were trimmed to the size of each region with reference to the outgroup sequences (ITS region included 5.8S) and phylogenetic analysis was performed using MEGA X [5]. The reference sequences of MO were obtained from the GenBank database (Table 3). Sequence information of *S. cerevisiae*, *Kluyveromyces nonfermentans*, and *K. marxianus* was used as the outgroup (Table 3). The sequences of the three regions were used to create a phylogenetic tree using the maximum likelihood method, as reported by Abdi-Hachesoo *et al*. [1].

The D1/D2 region, which is used for genus-level identification of pathogenic fungi, was analyzed in 18 samples; 16 of these samples matched the MO sequence in the database (GenBank accession no. AF350243.1, KX426595, and KX426594) and were classified into the same group. Two cockatiel samples (M55 and M69) showed a single nucleotide polymorphism and formed a new group (Table 4, Fig. 3A).

Analysis of 18 samples for the ITS regions, which are commonly used for the species-level identification of fungal pathogens, showed that MO isolated from two samples of Passeriformes and 15 samples of Psittaciformes belonged to group B, as reported by Abdi-Hachesoo *et al*. [1]. Only one sample (M60) from a zebra finch belonged to group A. In the study by Abdi-Hachesoo *et al*., group A consisted only of MO isolated from Passeriformes and group B consisted only of MO isolated from Psittaciformes [1]. However, in our study, MO isolated from Passeriformes was also included in group B, indicating that the groups were not completely separated by the taxonomic order of the host bird (Fig. 3B).

For the IGS1 region, which is suitable for identifying pathogenic fungi at the species and strain levels, 17 of the 18 samples analyzed were classified into the same group and only one sample (M60) was not included in this group (Fig. 3C).

Furthermore, the association of MO genotype with symptoms and morphology was evaluated. MO detected in eight birds with signs of gastrointestinal disorders and nine birds without such disorders (Table 1) belonged to the same group in all the analyses of the D1/D2, ITS, and IGS regions (Fig. 3). MO isolated from cockatiels (SuMO1) and budgerigars (SuMO2) with different morphologies were also genotyped (Fig. 1), and both belonged to the same group in all analyses (Fig. 3). Genotyping based on the D1/D2, ITS, and IGS regions did not distinguish between differences in the presence of gastrointestinal disorders and differences in morphology.

In this study, we established a method to obtain sequences directly from avian fecal samples for genotyping. Fecal samples are often contaminated by various PCR inhibitors and other fungi; therefore, the copy number of MO is relatively low. Although conventional PCR failed, seminested PCR using specific primers confirmed the amplification of the MO sequences in all samples due to its sensitivity and specificity, especially in contaminated materials. As fecal samples are easily obtainable and readily available, genotyping and molecular epidemiological studies of MO will be facilitated via our newly developed method described in this study.

ITS analysis revealed that most of the MO collected from Japanese pet birds belonged to a single group. Although it has been suggested that phylogenetic differentiation of the ITS1 region is related to the taxonomic order of the host [1, 9], our investigation refutes this notion. Genotyping based on the ITS regions did not distinguish between differences in the presence of gastrointestinal disorders and differences in morphology.

However, the ITS region is not suitable for differentiating between species and strains [13]; the IGS region, which is highly variable comparatively, is often used to analyze pathogenic fungi. For example, the differences in DNA sequences between the three mutants of *Cryptococcus neoformans* are 40–65% in the IGS region, as compared to those in the ITS region (approximately 1%) [10]. Furthermore, analysis of IGS sequences can detect the presence/absence of disease and reveal regional specificity. With respect to *Malassezia globosa* infections, for example, IGS sequences differ between patients and healthy individuals [12]; for *Trichosporon asahii*, regional specificity of clinical isolates has been revealed based on IGS sequences [11]. Thus, analysis of IGS sequences is common in pathogenic fungi, but not in the case for MO. However, genotyping based on the IGS1 region did not distinguish between differences in the presence of gastrointestinal disorders and differences in morphology.

The DNA sequence differences between the two genotypes found in this analysis of the ITS and IGS1 regions were 1.51% (8/529 bp) and 2.68% (11/411 bp), respectively, which were not highly variable regions. In conclusion, the ITS and IGS1 regions had little genetic variation and were not suitable for molecular epidemiological studies of MO. Accordingly, further studies are required, including a search for other gene regions.

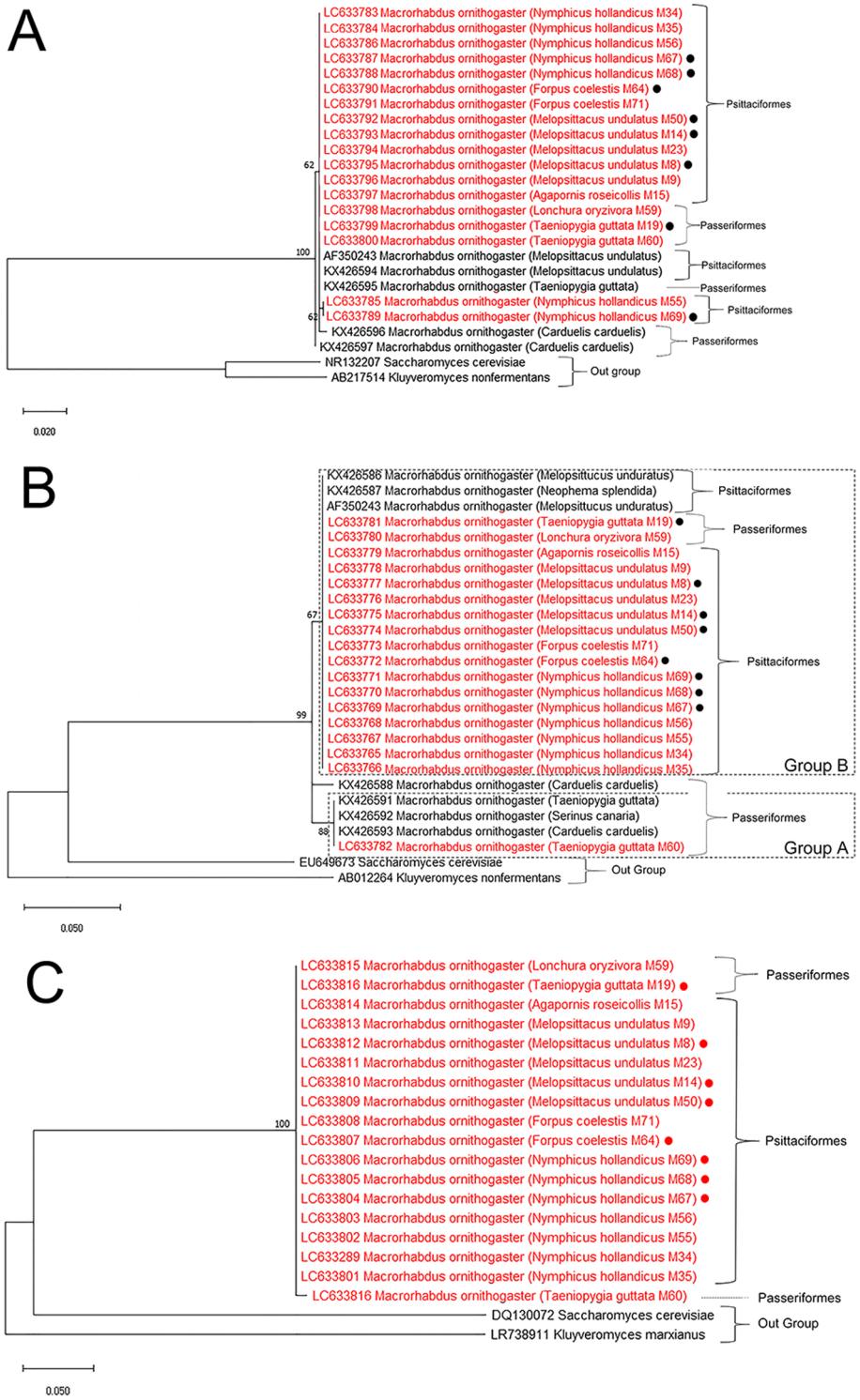

Fig. 3. Maximum likelihood tree of *Macrorhabdus ornithogaster* based on nucleotide sequences: (**A**) D1/D2, (**B**) internal transcribed spacer (ITS), and (**C**) intergenic spacer (IGS) regions. *Saccharomyces cerevisiae*, *Kluyveromyces nonfermentans*, and *K. marxianus* were analyzed together as an out-group. Bootstrap rate values are shown next to the branches. Accession number and bacterial name are followed by the scientific name of the sampled bird species in ( ). For samples analyzed in this study, the scientific name is followed by the sample number. In addition, samples of birds showing gastrointestinal symptoms are marked with ●, and the order name of the bird is marked with }. In (B), group A and B genotypes reported by Abdi-Hachesoo *et al*. are circled with dotted lines [1]. In the analysis of the D1/D2 region, 16 samples matched the *Macrorhabdus ornithogaster* (MO) sequence in the NCBI database (GenBank accession no. AF350243.1, KX426595, and KX426594) and were classified in the same group, while two samples from cockatiels (M55 and M69) formed a new group (A). Analysis of the ITS region showed that two MO samples of Passeriformes and 15 MO samples of Psittaciformes belonged to group B. Only one sample (M60) from a zebra finch belonged to group A. The groups were not separated by host bird order (B). In analysis of the IGS1 region, 17 samples were classified into the same group and only one sample (M60) was classified into a different group (C). No association with gastrointestinal symptoms was observed in any of the analyses.

CONFLICT OF INTEREST. The authors declare no conflicts of interest associated with this manuscript.

ACKNOWLEDGMENT. We thank Ms. Satoko Sugimoto for discussing the results and supporting the preparation of the manuscripts.

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