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Original Article

Molecular identification and phylogenetic analysis of *Macrorhabdus ornithogaster* based on the *18S rRNA* gene in companion birds of Tehran, Iran

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Abstract

Background: *Macrorhabdus ornithogaster* (*MO*) is an infectious yeast which can cause acute gastric disturbances in birds. It has a worldwide distribution with a broad host-range of bird species. **Aims:** Molecular identification and phylogenetic analysis of *MO* based on the *18S rRNA* gene in companion birds of Iran. **Methods:** A total of 54 stool samples were taken from birds (10 species) suspected of being infected. The presence of *MO* in collected stool samples was investigated using direct wet mount microscopy. Specific primers were designed to identify the *MO 18S rRNA* gene by using PCR. PCR products were sequenced and phylogenetic analysis was performed to determine the molecular diversity. **Results:** The obtained results demonstrated that 44.44% and 59.26% of the samples were diagnosed positive based on the first and second specific primers, respectively. *MO* was detected in the feces of canary, goldfinch, budgerigar, toucan, and English budge. Phylogenetic analysis revealed that *MO* sequence data from canaries, finches, and goldfinches had homology with an *MO* isolated from a German zebra finch. Moreover, *MO*s from cockatiels, rosy faced love birds, and budgerigars had a high phylogenetic similarity with multiple references, including American budgerigar, Japanese cockatiel, European goldfinch, and German budgerigar. **Conclusion:** *MO* exists in many species of Iranian birds, including goldfinches, budgerigars, toucans, and English budgies. As indicated by phylogenetic and polymorphism data analysis, the newly designed specific primers spanning a large portion of *18S rRNA* gene of *MO*, provides additional tool to detect and study the molecular diversity of *MO*.

Key words: *18S rRNA*, *Macrorhabdus ornithogaster*, PCR, Phylogenetic

Introduction

Macrorhabdus ornithogaster (*MO*) is a filamentous, rod-shaped yeast that exclusively colonizes at the junction of the proventriculus and ventriculus in birds. The vegetative cells divide by fission and are elongated, ranging from 2 to 20 µm in length. These cells can exist individually or in short chains, with visible nuclei at regular distances. Previously, *MO* was classified as a bacterial species, but further research by Tomaszewski *et al*. (2003) using *18S* and *26S rDNA* analysis revealed that it is, in fact, anamorphic ascomycetous yeast (Tomaszewski *et al*., 2003). Although it is Gram-positive, only the cytoplasm can be stained using the Gram-stain (Hannafusa *et al*., 2007; Borrelli *et al*., 2015; Sullivan *et al*., 2017). Other staining methods such as silver staining, periodic acid-Schiff staining, and hematoxylin and eosin staining can also be utilized for the detection of *MO* (Abdi-Hachesoo *et al*., 2019).

Different *MO* strains is found across the globe and has a broad spectrum of hosts, such as passerines, psittacines, chickens, turkeys, ostriches as well as other animals like dogs, cats, and laboratory mice (Cooke, 2000; Huchzermeyer and Henton, 2000; Martins *et al*., 2006; Robino *et al*., 2019). Birds infected with *MO* may develop macrorhabdosis, a condition that can cause acute gastric problems or gradual weight loss that may eventually result in death. Birds that look healthy can shed yeast cells into their environment without showing any signs of illness (Borrelli *et al*., 2015; Abdi-Hachesoo

et al., 2019; Kojima *et al*., 2022).

Macrorhabdosis can present with clinical signs such as vomiting, diarrhea, undigested seeds in feces, and chronic weight loss. Nevertheless, these symptoms are not exclusive to this condition and can also occur in other diseases (Ozmen, 2013; Püstow and Krautwald-Junghanns, 2017). Other potential diagnostic aids include radiographic imaging to identify an enlarged proventriculus, and the direct detection of the yeast through fecal wet smears. Despite the availability of effective antifungal drugs like Amphotericin B and Nystatin, controlling the disease caused by *MO* is challenging due to the poor conditions and high density in bird breeding and keeping centers. Consequently, eradicating the infectious agents such as *MO* is difficult. Therefore, there is an urgent need for a faster diagnostic method (Phalen, 2014; Baron *et al*., 2021).

Genetic characterization is considered the most reliable approach for diagnosing *MO* due to its remarkable accuracy and specificity. At present, the diagnosis of *MO* involves utilizing polymerase chain reaction (PCR) on tissue lesions located in the proventriculus and examining the bird's digestive system contents to validate the presence of the yeast (Kojima *et al*., 2022). However, the major drawback of this method is that sampling the proventriculus contents requires dissection of the birds. Hence, there is a need to devise new sampling techniques for swift detection.

In this study, we established a PCR-based method to specifically detect *MO* in avian feces. Next, we performed a phylogenetic analysis of *MO*s isolated from Iranian domestic birds to determine the origin of the yeast in different species of birds.

Materials and Methods

Sampling

Stool samples were collected individually from 54 suspicious birds that visited the "Little Bird Hospital" Pasteur Pet Clinic, Louisan Bird Park (Tehran, Iran) over of one year and the presence of *MO* was detected via direct wet mount microscopy. Clinical information was collected and a history form was prepared. Obtained samples were stored and frozen in a volume of 500 mg in a microtube.

DNA extraction

DNA extraction from obtained samples was carried out by Dyna Bio Blood/Tissue DNA extraction mini kit (Takapouzist, Iran) according to the kit's manufacturer instructions. The quality and amount of extracted DNA were evaluated by NanoDrop 2000c system (Termo Scientific, USA). Extracted DNA samples were kept at -20°C until further use in a nested PCR test.

Primer designing

The specific primer sequences used for the amplification of *18S rRNA* gene in *MO* are shown in Table 1. These primers were designed according to the obtained sequences of *MO* from NCBI database (https://ncbi.nlm.nih.gov/). Also, the primer blast tool of the NCBI database was used for checking the specificity of the designed primers. The expected size of the PCR products for the first and second pair of primers were 857 and 405 bp, respectively.

Nested PCR procedures

A commercial PCR master mix (Sinaclon Co., Tehran, Iran) was used for performing the PCR. All reactions were carried out in a final volume of 25 µL. For the first pair of primers, the PCR time-thermal condition was a pre-denatured at 95°C for 2 min, followed by 30 thermal cycles containing denaturation at 95°C for 30 s, annealing at 58.2°C for 30 s, and extension at 72°C for 60 s, and a final extension at 72°C for 5 min. A similar temperature program was used for the second primer pair, except for the annealing temperature that was 59.3°C.

Gel electrophoresis

PCR products were electrophoresed on a 1.5% agarose gel containing 10 µL of SYBR Green stain. The formed bands were then observed by an electrophoresis device (Syngene Bio Imaging, UK).

Sequence analysis

A total of 14 positive specimens were sequenced, and then the obtained sequences were aligned using BioEdit software (ver. 7.1). The sequences were blasted in NCBI database and were compared with other sequences which were previously registered in NCBI from different regions of the world. The levels of similarity and percentage of coverage among the sequences were investigated.

Phylogenetic analysis

The sequences of the reference strains of avian gastric yeast with the following accession numbers: KX426586, KX426588, AF350243, KX426589, LC647654, NG_063253 (*Hanseniaspora occidentalis*), and MH545924 (*Candida bracarensis*) were obtained from the NCBI Genomes database and were chosen for phylogenetic analysis, which was conducted using

Table 1: Primer sequences for the detection of *M. ornithogaster* in the stool samples that have been designed and utilized in this study

Primer name	Accession No.	Sequence	Location (nt)	PCR product size (bp)
Primer 1-F	AF350243.1	ACGAACGAGACCTTAACCTACT	1309-1713	405
Primer 1-R		TCTTCACTATAGAATGGAGTCGCC		
Primer 2-F	AF350243.1	AGGGCCTGTATAGGTCTTGT	475-1332	857
Primer 2-R		TAGTAGGTTAAGGTCTCGTTCGTTA		

MEGA 7 software (https://www.megasoftware.net/). A phylogenetic tree was constructed by the Neighbor-Joining method for each designed primer. Bootstrapping over 1000 replicates was done to assess the confidence level of the branch pattern.

Results

Direct wet mount microscopy

Among 54 obtained stool specimens, 23 samples including 2 goldfinches, 4 cockatiels, 10 domestic canaries, 2 rosy-faced lovebirds, 1 toucan, 2 budgerigars, 1 English budgie, and 1 finch were diagnosed positive based on the direct wet mount microscopy (without staining).

PCR and gel electrophoresis

Based on the PCR results, 24 out of 54 (44%) stool samples were positive for primer 1 and formed a clear band with a molecular weight of 857 bp after the gel electrophoresis. Also, 32 specimens (59%) were found to be positive for the second primer and formed a 405 bp band after the gel electrophoresis (Table 2).

Table 2: A summary of positive fecal samples in wet mount microscopy and PCR (specific primers 1 and 2)

Bird species	Number of obtained stool specimens	Number of positive wet mount specimens	Number of positive specimens for primer 1	Number of positive specimens for primer 2
Domestic canary	23	10 (43.48%)	7 (30.43%)	13 (56.52%)
Cockatiel	15	4 (26.67%)	8 (53.33%)	9 (60%)
Rosy-faced lovebird	5	2 (40%)	4 (80%)	4 (80%)
Goldfinch	4	2 (50%)	1 (25%)	1 (25%)
Budgerigar	2	2 (100%)	2 (100%)	2 (100%)
Toucan	1	1 (100%)	-	1 (100%)
English budgie	1	1 (100%)	1 (100%)	1 (100%)
Grey parrot	1	-	1 (100%)	1 (100%)
White-eared bulbul	1	-	-	-
Finch	1	1 (100%)	-	-
Total	54	23 (42.59%)	24 (44.44%)	32 (59.26)

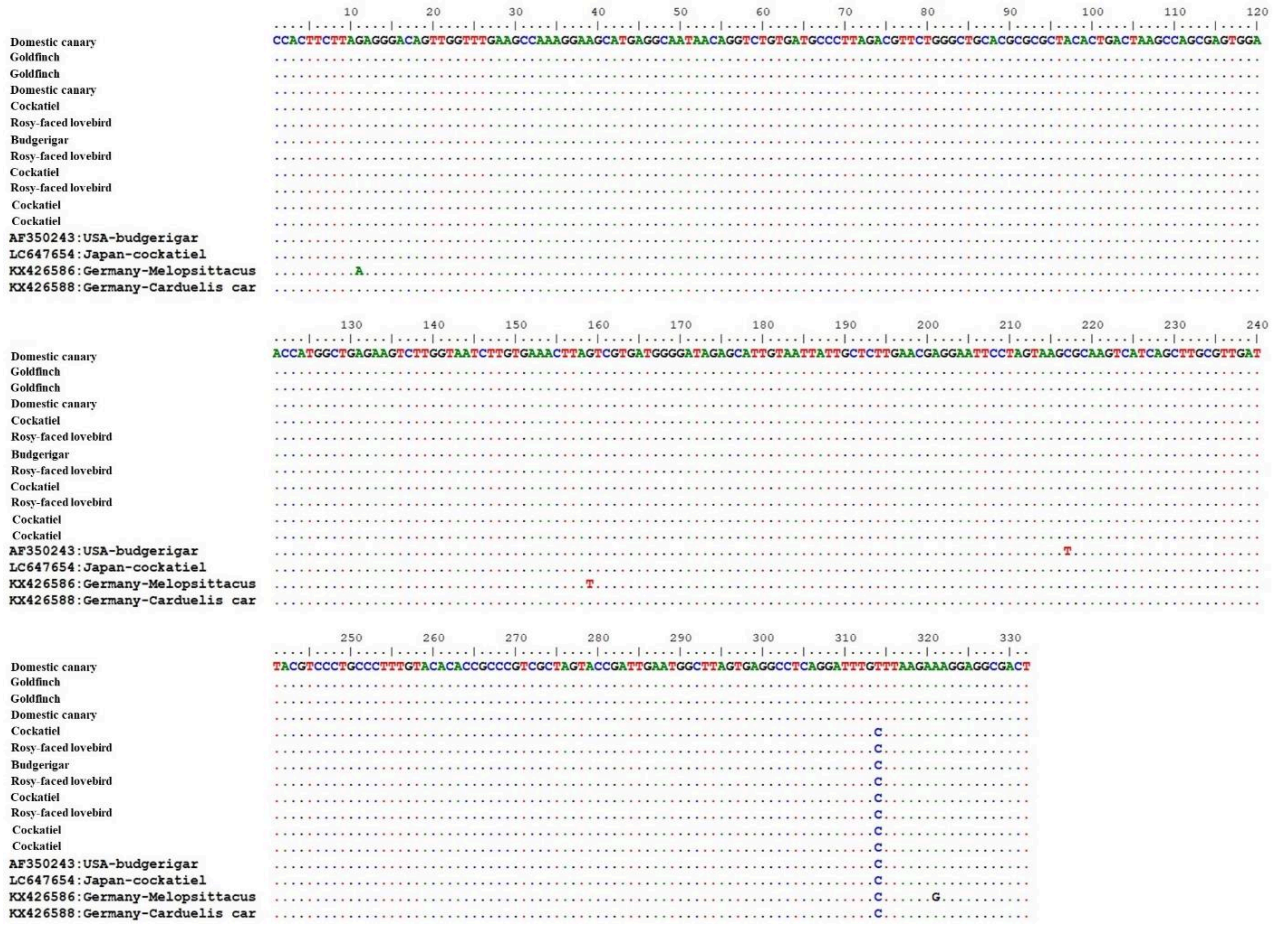

Fig. 1: Aligned sequences of the partial *18S rRNA* gene of *Macrorhabdus ornithogaster*, amplified by using the first designed primers. The sequences of local variants of Iran are shown aligned with the reference sequences obtained from GenBank

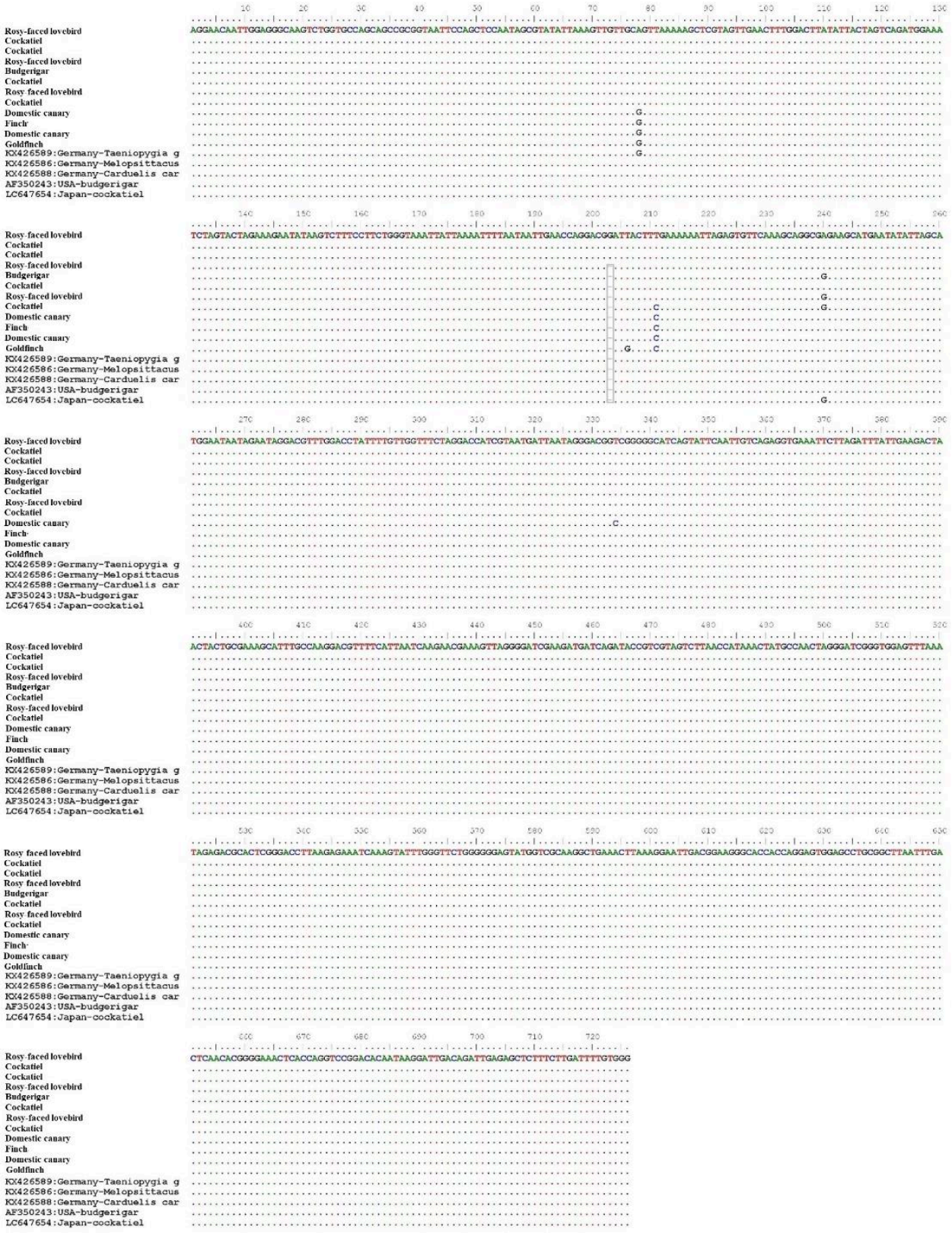

Fig. 2: Aligned sequences of the partial *18S rRNA* gene of *Macrorhabdus ornithogaster*, amplified by using the second designed primers. The sequences of local variants of Iran are shown aligned with the reference sequences obtained from GenBank

Sequence analysis

The results of the sequence analysis for the products of the first primer pair demonstrated that in all sequenced specimens compared to *MO* isolated from German budgerigar (KX426586), in positions 11 and 159 guanine was substituted for adenine and thymine, respectively. Moreover, in position 321 guanine was replaced by adenine. A similar comparison between *MO* isolated

from the American budgerigar (AF350243) and sequenced specimens revealed that in position 317 cytosine was substituted for thymine. Also, goldfinch and canary samples had thymine instead of cytosine in position 314. Aligned sequences are shown in Fig. 1.

The results of the sequence analysis for the products of the second primer pair showed that in canary, goldfinch, and finch specimens, similar to the *MO* of German zebra finch (KX426589) in the nucleotide 78 guanine was substituted for adenine (Fig. 2). In three of the samples (including one rosy-faced lovebird and two cockatiels), there was guanine in position 203, while in other samples and reference sequences, this position was a gap. Contrary to all other specimens, in goldfinch's nucleotide 206, guanine was observed instead of thymine. Also, in five samples, including two canaries, one cockatiel, one goldfinch, and one finch, in position 212 guanine was seen in place of thymine. In three samples, similar to the reference sequence of Japanese cockatiel *MO* (LC647654), in nucleotide 240 guanine was substituted for adenine. Finally, in one of the canary samples, in nucleotide 334 cytosine was substituted for thymine.

All sequences obtained and analyzed in this study were deposited in GenBank under accession numbers OR048103-OR048114 (for the first primers), and OR048115-OR048126 (for the second primers).

Phylogenetic analysis

For each primer, a phylogenetic tree was constructed from all positive samples. Utilized reference sequences were retrieved from the GenBank database and were as follows: American budgerigar *MO* (AF350243), Japanese cockatiel *MO* (LC647654), European goldfinch *MO* (KX426588), German budgerigar *MO* (KX426586), *Candida bracarensis* (MH545924), German zebra finch *MO* (KX426589), and *Hanseniaspora occidentalis* (NG_063253).

The constructed phylogenetic tree for the first primer consisted of 3 main clusters. Three of the cockatiel samples and 2 samples collected from rosy-faced lovebirds clustered together with the reference sequences of German budgerigar *MO* (KX426586), European goldfinch *MO* (KX426588), and American budgerigar *MO* (AF350243). *MO*s isolated from domestic canary, finch, and goldfinch clustered together with the reference sequence of German zebra finch (KX426589). One of the samples, taken from a budgerigar and another sample, isolated from a rosy faced lovebird segregated together with the reference sequence of LC647654. One of the samples, collected from a cockatiel and *Hanseniaspora occidentalis* (NG_063253) separated by a long branch which accounts for distinct amino acid substitution detected by sequence analysis (Fig. 3).

Based on the findings of the phylogenetic analysis of the second primer pair, goldfinch, and domestic canary samples formed a separate cluster from reference sequences. While, samples taken from cockatiels, rosy-faced lovebirds, and a budgerigar clustered together with the reference sequences of American budgerigar *MO* (AF350243), Japanese cockatiel *MO* (LC647654), and European goldfinch *MO* (KX426588). The German budgerigar *MO* (KX426586) and *Candida bracarensis* were found to be separated by a long branch in the phylogenetic tree, which can be attributed to the presence of distinct amino acid substitutions that were detected via sequence analysis (Fig. 4).

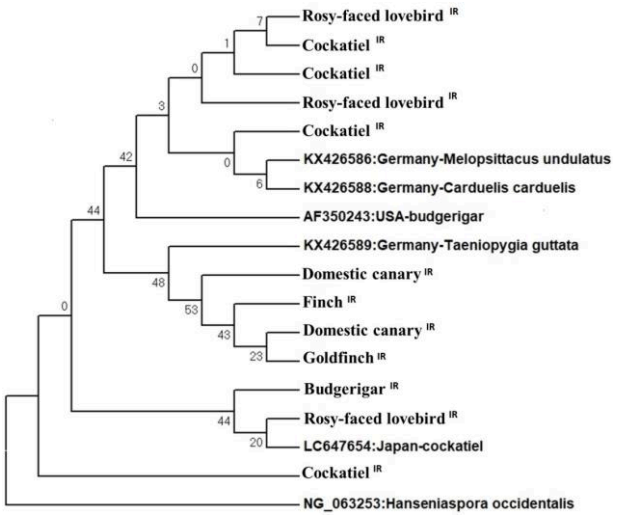

Fig. 3: Phylogenetic tree based on deduced amino acid sequence of the *18S rRNA* gene (specific primer 1). Reference sequences of *MO* were used for phylogenetic tree construction. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. IR: The abbreviation for the sequences identified in Iran

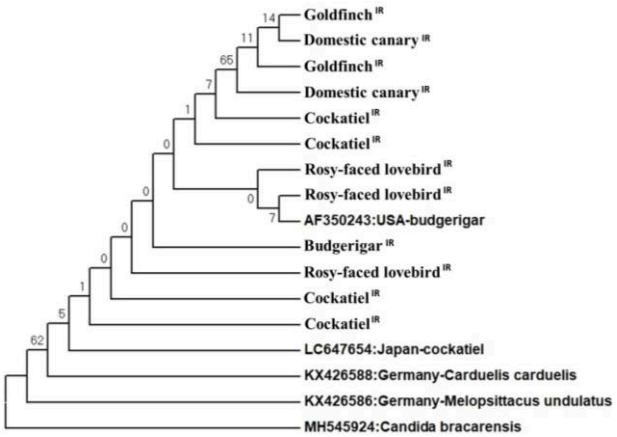

Fig. 4: Phylogenetic tree based on deduced amino acid sequence of the *18S rRNA* gene (specific primer 2). Reference sequences of *MO* were used for phylogenetic tree construction. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. IR: The abbreviation for the sequences identified in Iran

Discussion

Macrorhabdus ornithogaster is a pathogen that is

widely distributed across the globe and is known to affect different species of birds (Cooke, 2000; Huchzermeyer and Henton, 2000; Martins *et al*., 2006). Instances of *MO* infection have been reported in captive birds from Japan, North and South America, Europe, Australia, and Iran, indicating its global presence (Razmyar *et al*., 2016; Paula *et al*., 2018; Baron *et al*., 2019; Powers *et al*., 2019; Kojima *et al*., 2022). It seems that the yeast can infect not only birds, but also other animals such as dogs, cats, and laboratory mice, as there have been documented cases of *MO* isolation from these species (Cooke, 2000; Huchzermeyer and Henton, 2000).

The paper outlines the molecular detection of *MO* in birds referred to veterinary clinics. A technique was established to extract sequences directly from fecal samples, despite the presence of PCR inhibitors and other fungi which can reduce the *MO* copy number. PCR with specific primers was used to confirm the amplification of *MO* sequences in contaminated samples due to the high sensitivity and specificity of the method. The newly developed technique can facilitate genotyping and molecular epidemiological studies of *MO*, as fecal samples are easily accessible and available.

Based on the obtained results (Table 2), it seems that a high percentage of the evaluated birds had *MO* in their feces. Direct wet mount microscopy revealed that among the bird species from which at least 5 samples were taken, domestic canaries had the highest incidence of *MO* (10/23=43.48%). While rosy-faced lovebirds had the highest percentage of positive samples based on the results of the first and second specific primers (4/5=80% for both primers). Other Bird species such as goldfinches, budgerigars, toucans, English budgies, and finches were also diagnosed positive for the presence of *MO* in their feces based on the results of direct wet mount microscopy. In addition to the mentioned species, a sample taken from a grey parrot was also found to be positive for the presence of *MO* genetic material based on the results of the first and second specific primers. To the best of our knowledge, this study is the first research that confirms the presence of *MO* in the stool samples of goldfinches, budgerigars, toucans, and English budges in Iran.

The *MO* has been previously detected in companion birds of Iran and other countries (Lanzarot *et al*., 2013; Paula *et al*., 2018; Talazadeh *et al*., 2023). Since our sampling was from oriented birds and not properly random, our data could not be used for a prevalence study. It should be noted that the aim of this study was molecular identification and analysis based on the *18S rRNA* gene and not exactly the prevalence of *MO* in Iran. However, our results showed that the overall frequency of positive samples based on the first and second primer was 44.44% and 59.26%, respectively.

The main purpose of conducting a phylogenetic analysis was to explore the source of *MO* infection in different bird species. The outcomes of both constructed trees were mutually supportive. According to the results, it seems that *MO*s isolated from canaries, finches, and goldfinches have a high genetic similarity with German zebra finch *MO* (KX426589). Hence, the primers designed in this study, along with the *18S rRNA* gene, can be utilized in future studies for additional phylogenetic analyses of the *MO*s that will be collected from the three mentioned bird species.

Based on the results obtained from the two constructed phylogenetic trees, it appears that the *MO*s that were isolated from cockatiels, rosy faced love birds, and budgerigars share a significant similarity with several reference *MO*s, such as American budgerigar *MO* (AF350243), Japanese cockatiel *MO* (LC647654), European goldfinch *MO* (KX426588), and German budgerigar *MO* (KX426586). This phylogenetic similarity indicates the circulation of different strains of *MO* in the mentioned bird species. Hence, it's not possible to accurately determine the source of the *MO*s collected from the aforementioned bird species.

In conclusion, a specific PCR protocol was developed to detect *MO* in bird feces. Using this method, the presence of *MO* in the stool samples of several bird species was confirmed. Finally, phylogenetic analysis revealed that different strains of *MO* are circulating in Iranian companion birds.

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Conflict of interest

The authors declare that they have no competing interests.

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