Latin American Journal of Aquatic Research, 48(5): 758-767, 2020
DOI: 10.3856/vol48-issue5-fulltext-2500

Research Article

Isolation of actinomycetes from Nile tilapia (*Oreochromis niloticus*) cultivated in semi-intensive systems from Morelos, Mexico central zone

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ABSTRACT. The production of Nile tilapia (*Oreochromis niloticus*) has good technological development; however, today, it is still necessary to make it more efficient. One way to increase efficiency is to prevent disease and improve the food conversion factor. Since previous investigations of tilapia microbiota detected a high proportion of organisms belonging to the order Actinomycetes, this study was to isolate, identify, and describe the species of bacteria microbiota belonging to the cultured Nile tilapia. These were done with Nile tilapia grown in a warm sub-humid climate during spring and summer seasons. The biopsy of different organs was performed for bacteriological culture and 16S rRNA sequencing analysis. From the 180 tissue samples, 49 isolates of the order Actinomycetes were obtained, representing ten species from seven genera: *Microbacterium*, *Brevibacterium*, *Cellulomonas*, *Corynebacterium*, *Kocuria*, *Actinomyces*, and *Micrococcus*. In spring, *Microbacterium* dominated, accounting for 74% of the total population. In the summer, lower diversity was observed, with 39% represented by *Microbacterium*. 16S rRNA sequencing analysis enabled the classification of *Actinomyces neuii* and *Microbacterium lacticum* as *Kocuria varians* and *Agromyces indicus*; the classification of *Microbacterium imperiale* as *Rhodococcus* and *Micrococcus luteus* was confirmed. No sequences of *K. varians* have been reported in fish. *Microbacterium dextranoliticum* showed high similarity to environmental samples. Here is the first study that analyzes the bacteria population in tilapia at the genetic level with an ecosystem approach, present in healthy cultured tilapia, indicating their beneficial associations with the host, making them candidates as probiotics, among other possible functions, applicable in tilapia cultivation.

Keywords: *Oreochromis niloticus*; Nile tilapia; microbiota; Actinomycetes; seasonal variability; aquaculture

INTRODUCTION

In all systems of cultivation, Nile tilapia *Oreochromis niloticus* regularly interact with biotic and abiotic factors within the system, resulting in interspecific interrelations that can be beneficial or causing damage (Odum, 1971). Commensal bacteria promote the adaptive immune system in vertebrates (Ferguson *et al*., 2010; Standen *et al.*, 2015) and inhibit colonization by pathogenic bacteria either passively by competitive exclusion or actively by generating toxic secondary metabolites. Stress caused in fish by handling, sorting, grading, transport, and stocking influences the microbiota in different manners, with physiological, hormonal, and cellular effects on the host, contributing to disease development (Ley *et al.*, 2008).

A predominance of *Aeromonas*, *Micrococcus*, *Staphylococcus*, *Bacillus*, and *Pseudomonas* has been reported in the microflora of freshwater fish, including tilapia, which produce amylase and may facilitate the degradation of foods with a high content of elements from plants like starch. *Corynebacterium* forms such as Actinomycetes were present but were non-amylase producers (Sugita *et al*., 1997; Matin *et al*., 2019).

Bacteria in the order Actinomycetes are Gram-positive microorganisms with variable morphology (rods or cocci); are non-sporulated, catalase-positive, aerobic, or anaerobic; may exhibit motility; and mostly ferment glucose (Coyle & Lipsky, 1990). Soto-Rodríguez *et al.* (2013) reported the genus *Microbacterium*, Gram-positive bacteria with rod morphology, are pathogenic in juvenile Nile tilapia. Information re-

Corresponding editor: Jesús Ponce-Palafox

lated to microorganisms in the Actinomycetes in teleosts is limited. In previous studies of pathogenic bacteria associated with Nile tilapia, carried out in our laboratory, we observed a large number of Actinomycetes from the total population of bacteria. For this reason, it was interesting to know what type of species existed, their variation in the seasons of the year, and the population diversity of the order. Therefore, this study's objective was to isolate, identify, and describe the species of bacteria belonging to the cultured Nile tilapia microbiota by bacterial culture grown in warm sub-humid climate during the spring and summer seasons of 2015. This information has significant economic and social relevance to tilapia aquaculture in warm sub-humid climates and is necessary for describing the microbiota in cultivated *O. niloticus*.

MATERIALS AND METHODS

Study animals

The study was carried out in healthy Nile tilapia (*Oreochromis niloticus*) to analyze the order Actinomycetes' normal microbiota, with few organisms with 1 mm skin lacerations. Cultivated in semi-intensive systems (n = 30) during 2015 spring and summer seasons, in farms located in a central area of Mexico with an average annual temperature of 21.5°C (<10°C, >32°C).

Temperature and pH were measured, and sampling was performed using a random yarn at four different pond points. Five organisms were obtained per farm, three farms were sampled per season, size and weight measurements were recorded, and to visibly determine that fish were healthy external analysis of parasites or lesions were performed before necropsy and the collection of tissue samples. Because we worked on production farms and sampled other species for other studies within the same farm, producers could not provide us with more organisms for the present study.

Necropsy and tissue sampling

The animals collected were sacrificed by an overdose of MS222 tricaine methanesulfonate (0.16 g L^{-1}). The animals were cared for following the Guide for the Care and Use of Laboratory Animals, and the protocols used in this study were reviewed and approved by the Institutional Committee for Care and Use of the Experimentation Animals of the Faculty of Veterinary Medicine and Zootechnics CICUAE N°DC-2015-2-3 (CICUAE, for its acronym in Spanish). A biopsy of the gill, liver, spleen, and intestine was performed using sterile instruments; the samples were stored in 1.5 mL microfuge tubes at -20°C until analysis.

Bacteria isolation and identification

Each organ was macerated in a sterile saline solution (0.85% NaCl) in sterile individual bags. The homogenate was distributed with sterile swabs and incubated under aerobic conditions in duplicate at 37°C and room temperature (25-29°C) for 10 days on 5% blood agar and Eugon-enriched medium supplemented with 1% B supplement (hematin yeast extract, L-glutamine, coenzyme, cocarboxylase, dextrose) and 10% fetal bovine serum (Bohle *et al*., 2009). The growth of colonies was checked daily. After incubation, colonies were selected based on their shape, size, elevation, structure, color, and opacity and subcultured in 5% blood agar until pure cultures were obtained. The presumptive identification of the isolates was performed by primary bacterial identification and subsequent biochemical tests (MacFaddin & Jean, 2003).

The classification was determined using Cowan & Steel tax codes (Barrow & Feltham, 1993). According to the manufacturer, classifications obtained at the species level were corroborated using the api®Coryne microsystems from bioMérieux, Inc. (Marcy-l'Étoile, France) 's protocol. The results were analyzed using the electronic platform apiweb™ version: 1.3.1.

DNA extraction

A pure culture of each bacteria isolate was obtained using a Eugon-enriched medium containing 1% B supplement and 10% fetal bovine serum and incubation for 48 h. Bacteria DNA was obtained following the protocol described by Doyle & Doyle (1987). DNA quantity and quality were measured with a Nanodrop™ spectrometer (NanoDrop, Wilmington, DE, USA). The 16S rRNA fragment was amplified by PCR using the primers 8F (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1492R (5′ ACGGCT ACCTTGTTACGACTT 3′), in the synthesis unit of the biotechnology institute of the National Autonomous University of Mexico. PCR was conducted in a 50 µL final volume reaction containing 200 µmol L^{-1} dNTPs, 1.5 mmol L^{-1} mg, 1 µmol L^{-1} of each primer, 1X buffer, 1 U of the high-fidelity polymerase, and 100 ng of bacterial genomic DNA as a template. Samples were subjected to an initial cycle of denaturation (95°C, 5 min), followed by 35 cycles of denaturalization (94°C, 15 s), annealing (52°C, 15 s), and extension (72°C, 90 min), with a final extension step at 72°C for 5 min. Automated DNA sequencing was conducted in the synthesis unit of the biotechnology institute of the National Autonomous University of Mexico.

Molecular analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 10 (Kumar *et al*.,

2018). Electropherograms were analyzed, and the right and left extremes were removed. The 16S rRNA was generated from forward and reverse sequence data using aligner software. The evolutionary history was inferred using the maximum likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993).

Statistical analysis

Descriptive statistics as proportions and frequency were employed. The Kruskal-Wallis test was used to compare the isolation between organs. The Mann Whitney U test was used with GraphPad Prism v. 5.00 (GraphPad, La Jolla, CA, USA) to compare isolations between seasons.

RESULTS

Bacteria isolation and identification

No parasitic organisms were observed from the fresh analysis of the eyes, gills, mouth, and skin. In the analysis of 150 tissue samples, 19 isolates of the order Actinomycetes were obtained with 10 species grouped into five different genera: *Microbacterium*, *Brevibacterium*, *Corynebacterium*, *Actinomyces*, and *Micrococcus*. According to the season, 19 isolates were obtained in spring and 18 in summer, from a total of six farms analyzed. According to the parameters analyzed during sampling, the temperature was 28 ± 0.1°C and pH was 8.0 ± 0.2; the length and weight of the organisms were similar for both samples, with average weights of 79 ± 2 and 81 ± 2 g, and 14 ± 1 and 11 ± 1 cm in the spring and summer, respectively. Twenty-four different bacteria were found in the spring and 25 in the summer (Table 1).

Eighty percent of the isolates presented Gram-positive rod morphology in palisade and angles of 45°, while the remaining 20% showed Gram-positive cocci morphology with tetrad arrangement. In the genus *Microbacterium*, all isolates were catalase- and oxidase-positive, and *M. imperial* showed positive motility. *M. dextranoliticum* and *M. aurum* used glucose as a carbon source, and only *Micrococcus luteus* was urease-positive. All *Micrococcus* isolates reduced nitrite.

In the spring season, microorganisms of the genus *Microbacterium* were dominant, accounting for 74% of the total population (n = 15 spring), represented by 32% *M. dextranoliticum*, 37% *M. aurum*, and 5% *M. imperiale*. The genus *Brevibacterium* was the second most abundant group, followed by *Corynebacterium*, *Micrococcus*, and *Actinomyces* (Fig. 1a). In the summer season (n = 15 summer), lower genera of Actinomycetes were represented by *Microbacterium* (39%), *M. dextranoliticum* (11%), *M. aurum* (6%) and *M. lacticum* (22%). *Micrococcus luteus* represented 33% of the total population, followed by *Kocuria varians* (28%) (Fig. 1b).

Table 1. Total bacteria isolated from tilapia *Oreochromis niloticus*.

Season	Farm number	Number of bacteria per individual					Subtotal	Total
		1	2	3	4	5		
Spring	1	6	2	1	0	0	9	**24**
	2	4	3	0	0	0	7	
	3	5	1	2	0	0	8	
Summer	1	4	4	2	2	1	13	**25**
	2	2	0	2	1	3	8	
	3	0	2	0	1	1	4	

The gills contained a greater representation of Actinomycetes, followed by the intestine. No isolations were made from water samples. The bacteria present in gills are likely that they are similar to those found in the aquatic environment where they live (Lee, 2008; Tang *et al.*, 2008; Kumar *et al.*, 2013). In the spring season, the presence of Actinomycetes was greater in the gills, while in the summer, the intestine contained more Actinomycetes. No isolates were obtained in the spring in the spleen, with all isolates found in the summer season. The liver had the same number of isolates in each season. The present is the first report of this type of bacteria in organs such as the spleen and liver in Nile tilapia (*Oreochromis niloticus*); no apparent disease signs were observed. In the skin, samples collected in the spring season showed evident lesions; however, the number of isolates found was higher than those in the liver and spleen (Fig. 2).

There was a significant transition of the genera represented during the spring season, in which the most representative genus was *Microbacterium*, particularly *M. aurum* and *M. dextranoliticum* (Fig. 3a). During the summer, the abundance of *M. luteus* and *Kocuria varians* was higher, and *Microbacterium* was maintained, but *M. lacticum* was the most frequent (Fig. 3b). No significant differences were observed between the analyzed organs and the number of isolates found (P = 0.3743 for spring and P = 0.4593 for summer). In comparing tissues, no significant differences were observed between the spleen and gill ($P \leq 0.05$), although no spleen isolates were observed in the spring season.

No significant difference was observed when comparing the numbers of isolated strains between the spring and summer seasons ($P \leq 0.05$). A comparison of the number of isolates obtained using culture used revealed that the Eugon medium produced a higher number of isolates of species in the order Actinomycetes.

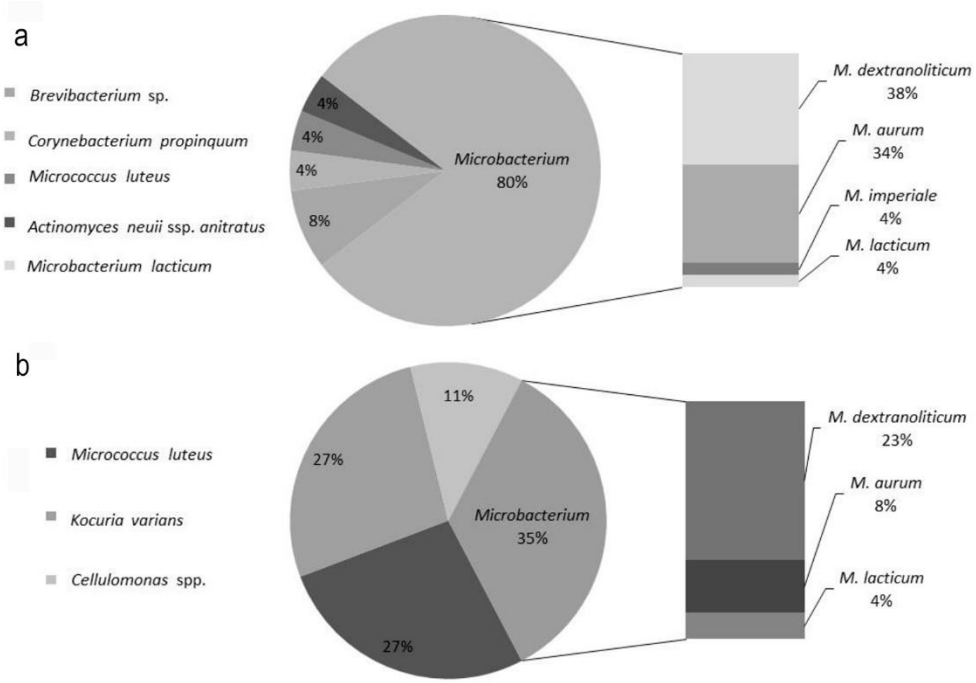

Figure 1. Diversity of Actinomycetes species isolated from tilapia in a) spring and b) summer. $P = 0.3743$ for spring and 0.4593 for summer.

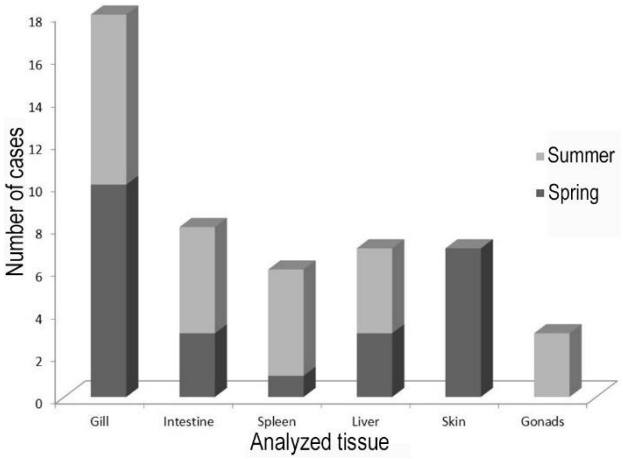

Figure 2. Distribution of Actinomycetes species in sampled organs (n = 15). Kruskal-Wallis test was performed with $P = 0.3743$ for spring and 0.4593 for summer.

Evaluation of these results by Fisher's test showed a significant difference ($P = 0.0076$) between the culture media used.

Molecular analysis

The taxonomic keys for bacteria and databases of the microsystems of identification are nourished with information mainly focused on human or veterinary medicine of terrestrial organisms, so identifying bacteria isolated from fish and aquatic environments may not be as accurate. Due to this, its identification with molecular tools and phylogeny was complemented. Species in the genus *Corynebacterium*, *Brevibacterium* and *Cellulomonas*, were observed in the same branch, with *Corynebacterium* and *Brevibacterium* then separated onto different nodes and into different taxa. These results indicate that these organisms can be classified as *Arthrobacter* (Genbank MN072343, MN077569, and MN072703) using genes as a reference, but it appears to be a different species or varieties. *Microbacterium lactcium* was observed in the same node that gave rise to *Microbacterium dextranoliticum*, indicating different but highly similar bacteria. For *Actinomyces neuii* subsp. *anitratus*, the genetic analysis suggested its classification as *Kocuria*, as shown for *K. varians* (MN072908). Finally, *Microbacterium imperiale* was classified separately from the other bacteria, and BLAST results revealed its classification in the genus *Rhodococcus* (MN072637) (Fig. 4).

Comparing the phylogeny of the 16S rRNA using a bootstrap test, including the first five queries (Fig. 5) and environmental samples (Fig. 6) of each bacteria analysis revealed that *Corynebacterium*, *Brevibacterium*, and *Cellulomonas* were classified as *Arthrobacter* sp. (MN072343, MN077569, and MN072703). In the case of *K. varians* (Genbank MN072929) and *M. luteus*

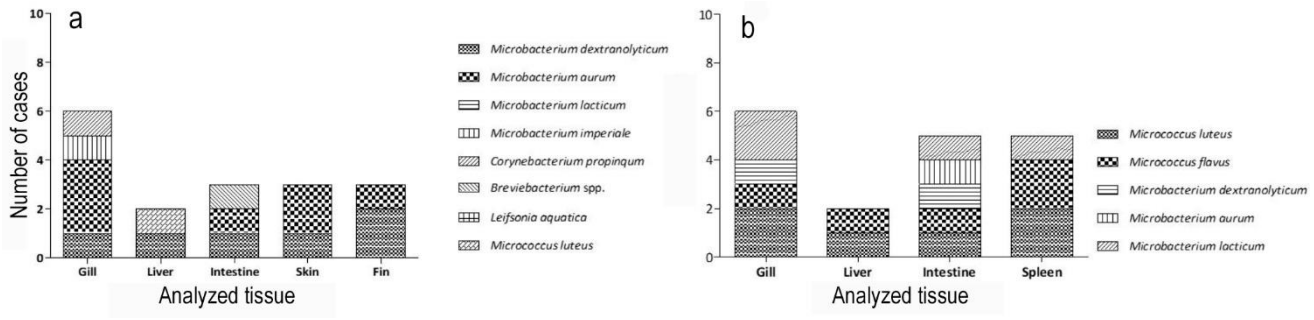

Figure 3. Relation of Actinomycetes isolated in the different Nile tilapia organs analyzed in a) spring and b) summer. Mann Whitney U test, with a value <0.05.

Figure 4. Molecular phylogenetic analysis by maximum likelihood method with 16S rRNA of bacteria. Ten nucleotide sequences representing the species isolated by traditional culture-based techniques were analyzed. There were a total of 1347 bases in the final dataset.

(Genbank MN071398), the identity was confirmed as along with *Microbacterium aurum* as *Microbac-terium phyllosphaerae* (MN075195). *Actinomyces neuii* were classified as *K. varians*. The genus of *M. dextranoliticum* was confirmed but differed in the species based on molecular analysis, which suggested *Microbacterium phyllosphaerae* (MN072639).

Using traditional culture base-techniques with the microsystems API/Biochemistry *versus* 16S rRNA analysis for tilapia Actinomycetes identification revealed different classifications for *Microbacterium* (Table 2). The API system database was created primarily from humans and some veterinary strains. The data in GenBank are limited concerning Actinomycetes fish bacteria, making species-level classification difficult.

DISCUSSION

The order Actinomycetes was represented by 10 different species in tilapia *Oreochromis niloticus*; the most representative genus in tilapia cultivated during the spring and summer seasons were *Microbacterium*. In the present study, different species of *Microbacterium* were isolated from different tissues such as the gill, liver, intestine, spleen, and gonad as well as skin containing cutaneous lesions. *Microbacterium aurum* was the most common species in the spring, accounting for 37 of 74% of the total population isolated, followed by the species *M. dextranoliticum* (32%) and *M. imperiale* (5%). In summer, species substitution was observed; the most represented species was *Microbacterium lacticum* (22%) followed by *M. dextranoliticum* (11%) and with a lower representation by *M. aurum* (6%). Species associated with skin lesions were *M. aurum* and *M. dextranoliticum*, as they were identified in the skin and tissue fins with signs of cutaneous ulceration (Funke *et al.*, 1995). Few studies have examined *Microbacterium* species in fish and have mainly been described as commensal microorganisms (Larsen *et al.*, 2013; Mansooreh *et al.*, 2015). However, Soto-Rodríguez *et al.* (2013) reported *M.*

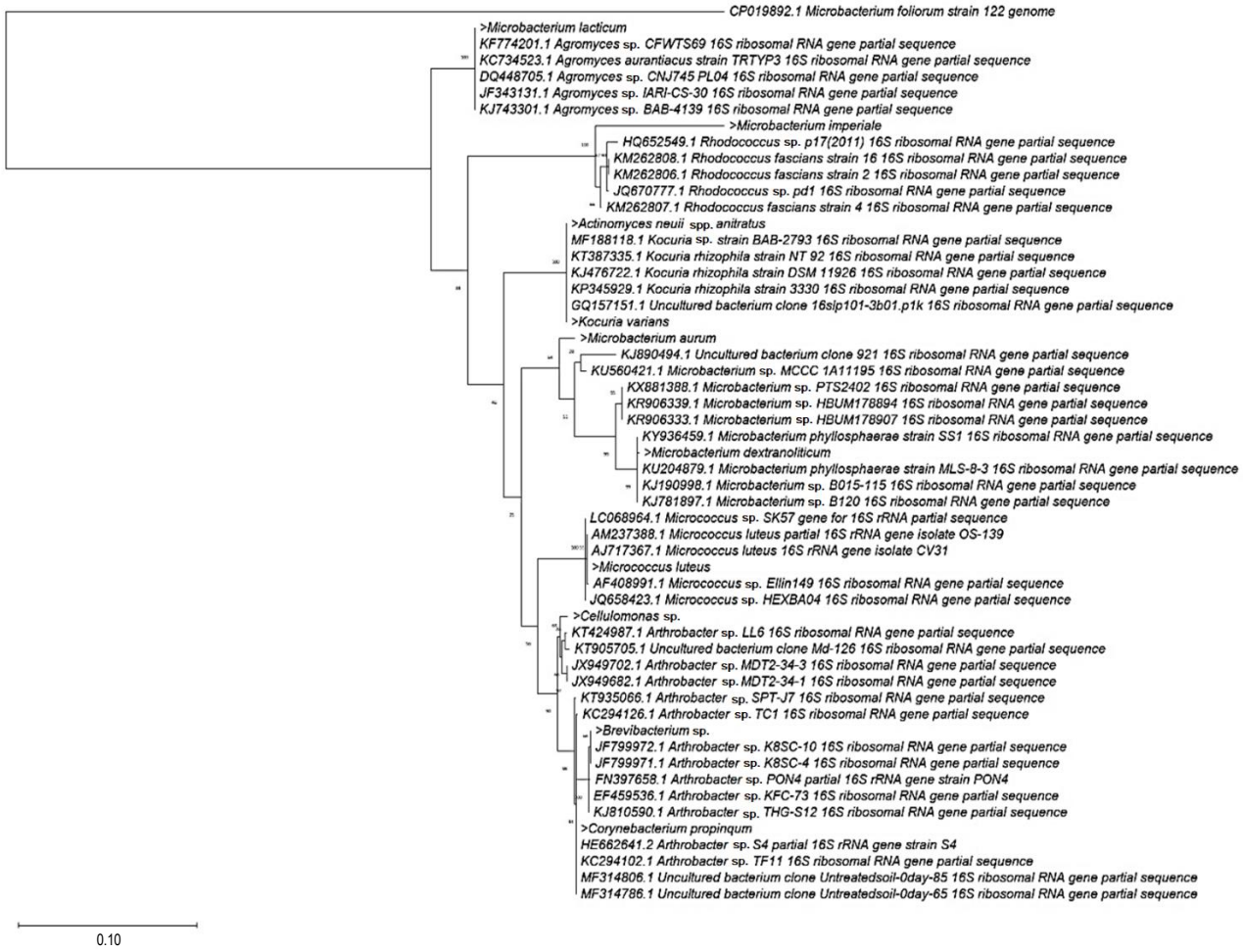

Figure 5. Molecular phylogenetic analysis by maximum likelihood method and bootstrap test phylogeny of the tilapia *Oreochromis niloticus* Actinomycetes 16S rRNA. Isolates examined in the present study were marked with a point and compared with each in the databank's first five queries. Fifty-five nucleotide sequences were analyzed, and 1135 bases were in the final dataset. Samples of the present study are indicated with the bullet >.

paraoxydans as a pathogen in cultured tilapia, which can be explained by the process of dysbiosis induced by external factors to the host such as changes in temperature, pH, and manipulation or overpopulation.

Species in the genera *Brevibacterium* and *Corynebacterium* have been reported as opportunistic pathogenic microorganisms in immunocompromised humans; however, these microorganisms are commonly found in sediments and water (Lee, 2008; Kumar *et al.*, 2013) and on normal skin and in the mucosal microbiota (Diez-Aguilar *et al.*, 2013), respectively. In contrast, there are no reports of pathogenicity in any teleosts for *Brevibacterium* spp. and *Corynebacterium propinquum*.

The genera *Brevibacterium* (5%), *Corynebacterium* (5%), and *Micrococcus* (5%) were equally represented in the spring. In the summer season, these genera were not represented equally in the total population, with *Micrococcus luteus* and *Kocuria varians* accounting for larger proportions (28 and 33%, respectively). However, the genus *Microbacterium* remained present at 39% of the total population analyzed in this season. Thus, the spring population is more diverse than the summer population.

Although it is known that *M. luteus* has probiotic potential in tilapia, the genus *Micrococcus* is poorly described in fish (Azza *et al.*, 2009; Standen *et al.*, 2015). In general, *Micrococcus* and *Microbacterium* show significant potential for use in aquaculture fish as probiotics and the production of secondary metabolites for the industry. *K. varians* has been detected in activated sludge, semen, and urogenital area of some freshwater fishes (Boonthai *et al.*, 2016).

Few studies have conducted a molecular analysis of the 16S rRNA based on information in the databank. Few

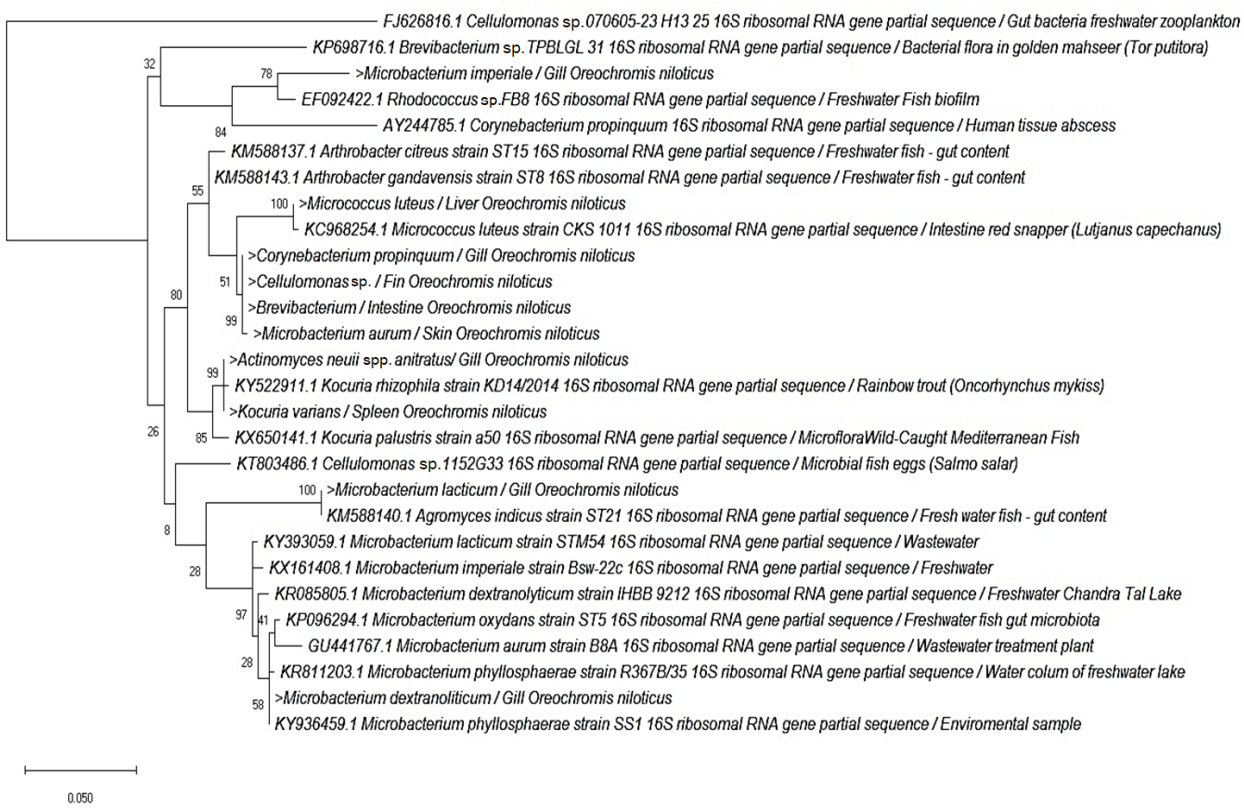

Figure 6. Molecular phylogenetic analysis by maximum likelihood method and bootstrap test phylogeny compares the present study bacteria 16S rRNA *versus* 16S rRNA of related bacteria with fish or freshwater environmental samples. The analysis involved 28 nucleotide sequences. There was a total of 400 bases in the final dataset. Samples from the present study are indicated with the bullet >.

Corynebacterium, *Cellulomonas*, and *Brevibacterium* have been described in freshwater fish or environmentally related samples, and there are no reports of *C. propinquum* in fish. For *Cellulomonas* sp. FJ626816.1 16S rRNA and 070605-23_H13_25, there are only two queries from gut bacteria samples of freshwater zooplankton species, but no phylogenetic relationship with *Cellulomonas* bacteria was observed in the present study (Fig. 6). For *Brevibacterium* sp., there is a report with accession number KP698716.1 of bacterial microbiota in golden mahseer (*Tor putitora*). Still, this species did not show a phylogenetic relationship with the strain in this study. For these three cases, the genetic analysis suggested classification as *Arthrobacter* sp. The query with the best identification and coverage was not from fish or environmental samples (Fig. 5), possibly because of the lack of genetic descriptions of freshwater fish bacteria in the genus *Arthrobacter*, which is important in fish studies.

Classification of the bacteria *M. luteus* was confirmed by 16S rRNA analysis. There are no reports of the *K. varians* 16S rRNA in fish. Thus, this is the first report of this species isolated in the freshwater fish *O. niloticus*. *Microbacterium dextranoliticum* showed high similarity to *M. phyllosphaerae* (KY936459.1) in environmental samples and a small difference from *M. phyllosphaerae* (KR811203.1) from freshwater lake columns, as well as a larger difference from *M. dextranoliticum* (IHBB9212) of freshwater Chandra Tal Lake. Still, there are no reports for freshwater fish (Figs. 5-6).

For the bacteria classified based on morphology and biochemistry as *Actinomyces neuii* subsp. *anitratus* and *M. lacticum*, 16S rRNA analysis revealed classifications as *K. varians* and *Agromyces indicus* (KM588140.1), respectively, based on their high phylogenetic similarity and percentage of identity and coverage to the genus *Agromyces* (Table 2, Fig. 6). 16S rRNA analysis of *M. imperiale* suggested a *Rhodococcus* bacteria, although it is important to consider that BLAST analysis results showed 93% identity and 91% coverage; the next query with classification at the species level was *Rhodococcus fascians*, a plant pathogen bacterium, with 93% identity and 90% coverage. This result and those of phylogenetic analysis (Fig. 6) suggest that the bacteria

Table 2. Comparison results of bacteria classification obtained using classical methods and molecular biology methods. *The system API was used for the classification getting the result up to the level of genus and species were established according to (Funke *et al*., 1995).

API/Biochemistry	API ID (%)	Databank access number	ID (%)	Coverage (%)
*Microbacterium dextranoliticum**	99.9	*Microbacterium phyllosphaerae* KY936459.1	99	99
*Microbacterium aurum**	99.9	*Microbacterium* sp. KU560421.1	98	96
*Microbacterium lacticum**	99.9	*Agromyces* sp. KF774201.1	99	99
Microbacterium imperial	99.7	*Rhodococcus* sp. HQ652549.1	93	91
Corynebacterium propinquum	96.9	*Arthrobacter* sp. HE662641.2	99	96
Brevibacterium sp.	99.2	*Arthrobacter* sp. FN397658.1	99	99
Actinomyces neuii ssp. *anitratus*	97.5	*Kocuria* sp. MF188118.1	100	99
*Micrococcus luteus**	99.9	*Micrococcus luteus* AM237388.1	99	99
Kocuria varians	99.9	*Kocuria* sp. MF188118.1	99	99
Cellulomonas sp.	99.9	*Arthrobacter* sp. KT424987.1	99	93

classified as *M. imperiale* with morphological and biochemistry analysis may be *Rhodococcus* sp. because of its phylogenetic differences from the *M. imperiale* strain Bsw-22c in freshwater samples (KX161408.1) (Fig. 6).

Most of the genera of the order Actinomycetes described in this study as part of the population of bacteria in Nile tilapia have been described in other species and environments as symbiont and commensal organisms (Dsouza *et al.*, 2015; Mansooreh *et al.*, 2015; Soonthornchai *et al.*, 2015; Kumar & Jadeja, 2018). Their presence may be related to the fish's state of health, probably regulating pathogen and opportunistic species. However, it is necessary to do more studies to verify this function within the ecosystem.

The present is the first report of Actinomycetes in Nile tilapia *O. niloticus*, contributing information to databases of bacterial genes of interest for aquaculture and enabling identifying bacteria in tilapia in the future. Analyzed tilapia shows a dominance of the genus *Microbacterium* with 80% in spring and 35% in summer. Being the gills, the organ that is most exposed to the external environment and the skin and tissue cavities' shows the highest percentage of isolates with 43%. The bacteria species with the lowest representation are *M. imperiale* and *M. lacticum*, with a 4% representativeness each. Although the bacteria were characterized in tilapia without signs of disease, in the two analyzed seasons, there are variations in the proportion of different genera of bacteria in the total population, which suggests a dynamism of the microbiota throughout the productive cycle. The analyzed culture tilapia presents strains such as *Brevibacterium*, *M. luteus*, *C. propinqum*, and *A. neuii* that only appear in spring. The *K. varians* and *Cellulomonas* species only in summer. With increases in the development of tilapia aquaculture in sub-humid warm climates, it is essential to identify all organisms associated with the fist to improve cultivation and prevent disease and mortality, thus avoiding antibiotics and the generation of resistant microorganisms.

ACKNOWLEDGMENTS

We acknowledge the advice and review of Dr. Carolina Tafalla Piñeiro and Dr. Estefania Muñoz Atienza from the INIA, Madrid, Spain. The MVZ Ana Lilia del Monte Rodriguez from the CENID-SAI, INIFAP Mexico, for technical assistance and the State Committee of Aquaculture Health of the State of Morelos, Mexico, for fieldwork and technical assistance. This work was supported by the National Council for Science and Technology (CONACyT) [grant #391616 2014-2018].

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Received: 20 January 2020; Accepted: 10 June 2020