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IDENTIFICATION OF *Bacteroides* spp. FROM DUCKS USING 16S rRNA GENE PCR ASSAY: PRELUDE TO ITS APPLICATION IN MICROBIAL SOURCE TRACKING

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ABSTRACT

Fecal pollution monitoring using fecal indicator bacteria has many limitations. Alternatively, using 16S rRNA gene *Bacteroides*-based polymerase chain reaction (PCR) showed promise because *Bacteroides* spp. are a major part of the gut microbiota. In the Philippines, duck backyard farming waste management is unmonitored. Hence, the objective of this study was to isolate *Bacteroides* DNA from duck feces and to establish genetic relatedness that can be used to develop new microbial source tracking markers. Fifty (50) duck fecal samples were collected. Of the 50, 20 were subjected to total DNA extraction and the remaining 30 were used for the isolation of *Bacteroides*. A total of 32 *Bacteroides* were isolated that included 6 *B. nordii* and 26 *B. ovatus*. A phylogenetic tree showing the host sources of *Bacteroides* generated five clusters. Four clusters came from this study, while the fifth cluster was composed of sequences from a study in Japan. This shows an intraspecies difference between *Bacteroides* species in the Philippines and Japan. This difference is a result of variations in the gut microbiome such as host species, diet, and geography. Results of this study will expand the present sequence database of *Bacteroides* from ducks that can be used in identifying sources of fecal pollution in the environment.

Keywords: *Bacteroides*, culture, phylogenetic analysis, polymerase chain reaction, 16S rRNA gene

INTRODUCTION

Water monitoring of fecal indicator bacteria (FIB) such as *Escherichia coli* and enterococci, is routinely used for determining levels of fecal pollution. However, the use of FIB has its limitations: (1) the major gut microbiota are not fecal coliforms but obligate anaerobes such as *Bacteroides* spp. and clostridia; (2) although *E. coli* shows specificity to fecal sources of contamination, the same cannot be said for other fecal coliforms since there are coliforms of environmental origin; (3) the presence of FIB exhibits poor correlation with the presence of pathogens in contaminated water, thus providing erroneous information; and (4) the presence of FIB does not indicate the origin of the fecal pollutant. If the origin of fecal contamination is correctly identified, more efficient mitigation plans can be put in place (**Savichtcheva and Okabe, 2006; Oun *et al.*, 2017**).

In view of these limitations of FIB-based monitoring, a new method known as microbial source tracking (MST) was developed. MST is based on the principle that there are fecal microorganisms that are more strongly associated with their specific hosts and thus may be used to indicate host-specific contamination (**Waso *et al.*, 2018**). MST has been used in many countries to determine and monitor the water quality of several large bodies of water (**Staley *et al.*, 2016; Symonds *et al.*, 2017; Jardé *et al.*, 2018; Jeong *et al.*, 2019**).

Among the MST methods developed, the most commonly used is the library–independent method (LIM). This method is based on the detection of host-specific genetic biomarkers (e.g. 16S rRNA and virulence genes). LIM goes by the concept that genetic biomarker sequences are not only specific to fecal bacteria, such as *Bacteroides* spp., but also specific to the host species, thus allowing discrimination among suspected sources (**Bernhard and Field, 2000; Rivera and Rock, 2011**). The most established MST biomarkers are *Bacteroides*-based biomarkers. *Bacteroides* spp. are the most diverse group of microorganisms in the gut of most animals. They have shown to have very high level of genetic host-specificity because they tend to coevolve highly with their hosts (**Bernhard and Field, 2000**). Additionally, there have been several *Bacteroides*-based MST biomarkers developed to discriminate human fecal sources from other fecal animal sources. These *Bacteroides*-based MST biomarkers were designed to target specific diagnostic sequences within the *Bacteroides* 16S rRNA gene present in feces from various animals (**Layton *et al.*, 2006**). Some of the widely used *Bacteroides*-based MST biomarkers are for humans (*HF183, HuBac, BacHum, BacH*), pigs (*Pig-1-Bac, Pig-2-Bac*), cows (*BoBac, BacBov, BacCow*), dogs (*BacCan*), geese (CGOF1-Bac, CGOF2-Bac), muskrats (*MuBa01*), and ducks (*DuckBac*) (**Kobayashi *et al.*, 2013; Koraijkic *et al.*, 2014**). Each of these MST biomarkers has shown varying degrees of sensitivity and specificity based on where the samples were collected. Hence, the need to establish MST biomarkers that would target more specific areas within the target sequence for better accuracy. The necessity to create new MST biomarkers has become even more apparent because the gut microbiome communities exhibit variations across different countries and latitudes (**Yatsuneko *et al.*, 2012; Suzuki and Worobey, 2014**). For example, human MST biomarker *HF183* was the best performing biomarker in the United States (California), France, Ireland, Portugal, the United Kingdom, Australia (Queensland), and Bangladesh, but it was the human MST biomarker *BacHum* that was found to be the best for Kenya and India. By contrast, human MST biomarker *B. theta* was the best performing biomarker in Darwin, Australia (**Gawler *et al.*, 2007; Ahmed *et al.*, 2008; Jenkins *et al.*, 2009**). Hence, even places within the same geographical area (Europe [i.e. UK and Ireland] and Australia [Queensland and Darwin]) would still show differences in the performance of MST biomarkers.

Therefore, the objective of this study was to determine the genetic relatedness of *Bacteroides* spp. (uncultured and cultured) from ducks. The results of this study will expand the present sequence database of *Bacteroides* from ducks that can be used in identifying sources of fecal pollution in the environment. This database can also be used in the development of more specific duck-associated MST biomarkers that will better differentiate duck *Bacteroides* in the Philippines and other locations that share similar duck farming condition. This study is limited to collection of droppings from farmed ducks. Moreover, all fecal samples were collected from caged ducks, although free-ranged duck farming is also common in the Philippines.

MATERIALS AND METHODS

Fecal sample collection

A total of 61 fecal samples were collected between February 2019 and June 2019 from the province of Laguna and Metro Manila in the Philippines. Fecal samples were collected from ducks (n = 50), chickens (n = 5), cows (n = 3), and dogs (n = 3). Freshly collected feces were transported on ice to the laboratory within 24 h. All samples were stored at -20 °C until use.
Duck fecal samples were collected from those found within the duck cages. Care was taken to make sure that there was no cross-contamination of fecal samples. With the assistance of farm workers, when a duck dropped feces, we were immediately informed to collect them at once. The same procedure was done in collecting chicken and cow droppings from poultry houses and cow farms, respectively. Dog droppings were collected from a local pet shop.

Total DNA extraction from fecal samples

Genomic DNA were extracted directly from 20 fecal samples of ducks, 5 from chickens, 3 from cows, and 3 from dogs using Qiagen QIAamp Stool Mini-kit (Qiagen Sciences, MD, USA) following the manufacturer's protocol. All extracted DNA were stored at -20 °C until use.

Culture and isolation of *Bacteroides* spp.

The remaining 30 duck fecal samples were used for the isolation and culture of *Bacteroides* spp. Decimal serial dilution was done by adding 1 g of fecal sample to 9 ml of normal saline water (0.9% NaCl). Of the first two dilutions (10^{-1} and 10^{-2}), 0.1 ml aliquots were spread plated in duplicates using *Bacteroides* Bile Esculin (BBE) agar (Laboratories Conda S.A., Spain). Plates were incubated for 4 days at 37 °C in an anaerobic condition using the Thermo Scientific™ Anaeropack™ anaerobic gas generator (ThermoFisher Scientific, USA). Preliminary indication of growth was observed when the brownish agar color of BBE agar turned to black. Distinct gray, circular, raised colonies were selected and re-streaked into fresh BBE agar for purification and incubated at 37 °C for 3–4 days in an anaerobic condition. Streaking for isolation, well-isolated colonies were sub-cultured every 3 weeks.
Extraction of DNA from pure *Bacteroides* cultures was done using the boiling method (**Garcia *et al.*, 2015**). The resulting suspension was vortexed and placed in a dry bath at 100 °C for 15 min. The resulting DNA extracts were kept at -20 °C until use.

Polymerase chain reaction (PCR)

The amplification of *Bacteroides* 16S rRNA gene was done using *Bacteroides*-specific primer pairs, Bac32F (5'-AAC-GCT-AGCTAC-AGG-CTT-3') and Bac708R (5'-CAA-TCG-GAG-TTC-TTC-GTG-3') (**Bernhard and Field, 2000**). The 30 µL PCR mixture contained 2X GoTaq Green Mastermix (Promega Corporation, Madison, WI, USA), 0.5 mM of each primer, 3 µL of DNA template and sterile nuclease-free water. The cycling conditions were as follows: 98 °C for 2 min, followed by 35 cycles of 95 °C for 30 sec, 56 °C for 1 min, and 72 °C for 1 min and a final extension of 72 °C for 5 min. PCR products were separated using 1% (w/v) agarose gel electrophoresis and visualized with staining by SYBR Safe DNA gel stain (Invitrogen, ThermoFisher Scientific, USA) under UV illumination.

Sequencing and phylogenetic analyses

PCR amplicons were sent to Macrogen, South Korea for purification and sequencing. Partial sequences (670 bp) were compared to similar sequences that are available in the National Center for Biotechnology Information (NCBI) using a BLAST Search. Sequences were aligned using MEGA X (Version 10.0.4). Additional *Bacteroides* sequences from other ducks were downloaded from GenBank with accession numbers AB666138, AB666140, AB666141, AB666152, and AB666154.
Chimera sequences in the partial 16S rRNA gene sequences of *Bacteroides* were checked using Decipher version 2.11.03 (**Wright *et al.*, 2012**) and rectified. Afterward, sequences were submitted to GenBank and were assigned the corresponding GenBank accession numbers.
Using MEGA X (Version 10.0.4), a phylogenetic tree was constructed using the Neighbor-Joining (NJ) method. Bootstrap analyses of 1000 replicates (Tamura-Nei) were done to approximate the confidence of the trees. The sequence of *Phocaecola abscessus* (EU694176), a species within the family *Bacteroidaceae* to which *Bacteroides* also belongs, was used as an outgroup.

RESULTS

Isolation and molecular identification of *Bacteroides* spp.

A total of 32 presumptive *Bacteroides* isolates were grown in culture using BBE agar at an anaerobic condition. All isolates consistently exhibited a gray, raised, and circular colonies with a change in agar color from brown to black indicative of the biochemical reaction of *Bacteroides* to esculin (Figure 1).

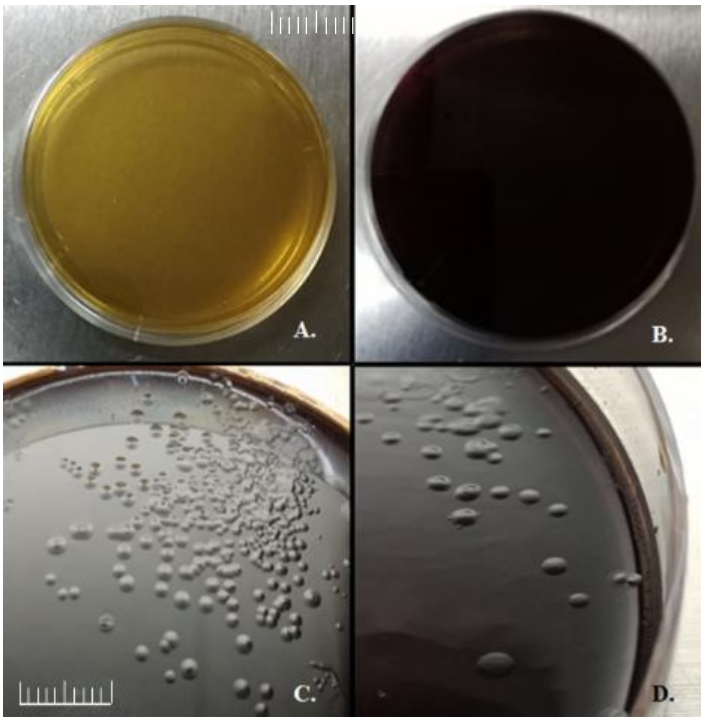

Figure 1 *Bacteroides* cultivation in *Bacteroides* Bile Esculin (BBE) Agar. (A) Uninoculated BBE Agar (underside view); (B) Inoculated BBE Agar (underside view); (C - D) Distinct *Bacteroides* colony growth in BBE Agar. Bar Scale: 10 mm

On the other hand, of the 20 fecal samples collected for total DNA extraction and 16S rRNA gene amplification, only 15 samples were shown to have amplified the *Bacteroides* gene sequences using *Bacteroides*-specific primers. All the other taxonomic Orders shown in Figure 2 are taxonomic Families within the Order Bacteroidetes. Also, as shown in Figure 2, all 15 samples that were amplified have clustered with the representative species of *Bacteroides* in the Family *Bacteroidaceae* (*B. nordii, B. fragilis, B. thetaiotamicron, B. finegoldii, B. ovatus*) with high boostrap values.

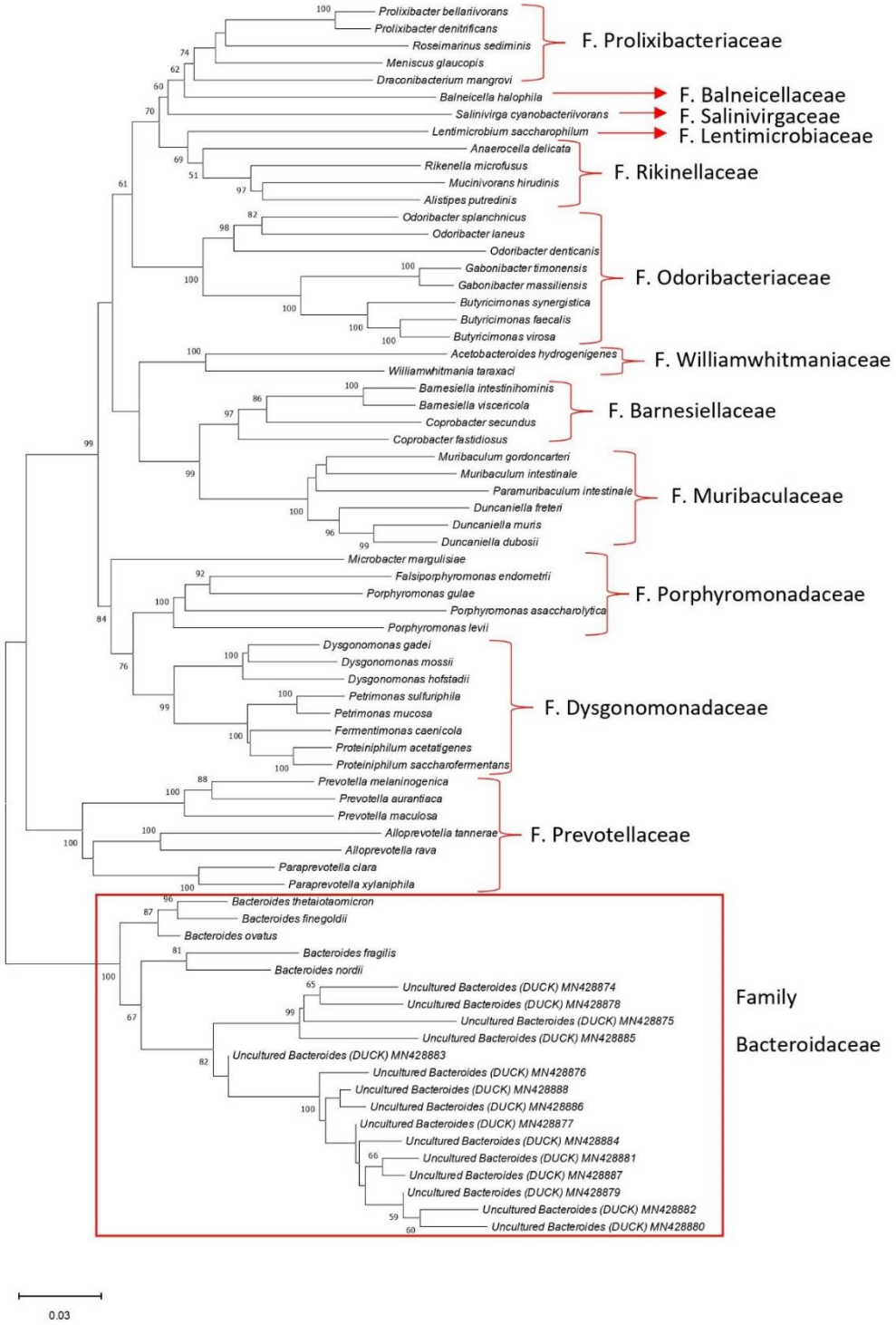

Figure 2 Phylogenetic relationships based on the partial 16S rRNA gene sequences acquired from duck fecal samples using Neighbor-Joining (NJ) method at 1000 replicates together with downloaded sequences of other families under Order Bacteroidetes. Only values >60% are shown.

All 32 isolates were identified as *Bacteroides* when cross-referenced with the NCBI database. A total of 6 out of 32 *Bacteroides* from farmed duck feces were identified to belong to *B. nordii* with acceptable % similarities of 99.35%–99.84%. This was supported by the phylogenetic tree (Figure 3) showing that the NCBI-identified *B. nordii* did group with the reference sequence of *B. nordii* with high bootstrap value of 99%. On the other hand, 26 of the 32 isolates were identified as *B. ovatus* (97%–99%). In the constructed phylogenetic tree (Figure 3), all the isolates identified through NCBI as *B. ovatus* formed low bootstrap values to the reference sequence of *B. ovatus*. Thus, in such case of high NCBI percent similarity but showed low bootstrap values to the reference strain, the 26 *Bacteroides* isolates were simply registered and submitted to NCBI as *Bacteroides* spp.

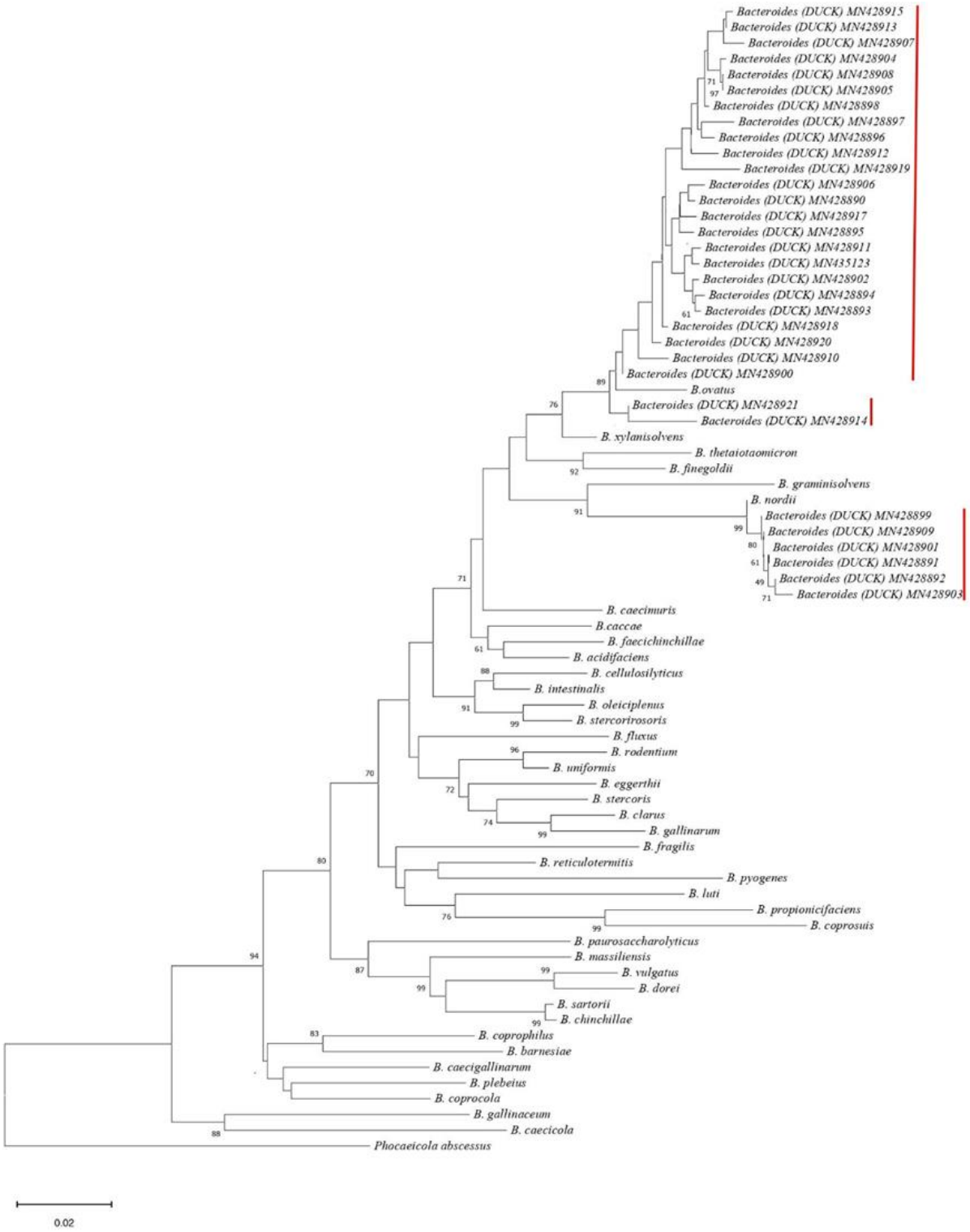

Figure 3 Phylogenetic relationships of 32 *Bacteroides* isolates from duck feces using 16S rRNA gene sequences with reference sequences of valid *Bacteorides* species using Neighbor-Joining (NJ) at 1000 replicates. Values >60% are only shown. *Phocaeicola abscessus* was used as an outgroup.

Duck (host)-origin phylogenetic analysis

The 16S rRNA gene, from the extracted total DNA of duck fecal smaples and *Bacteroides* isolates, were amplified and sequenced. Additional 16S rRNA gene sequences were downloaded from NCBI such as those from humans, cows, pigs, chickens, goats, and fish. The phylogenetic tree shows that the sequences clustered into several clades representing *Bacteroides* species coming from different animal sources (Figure 4).

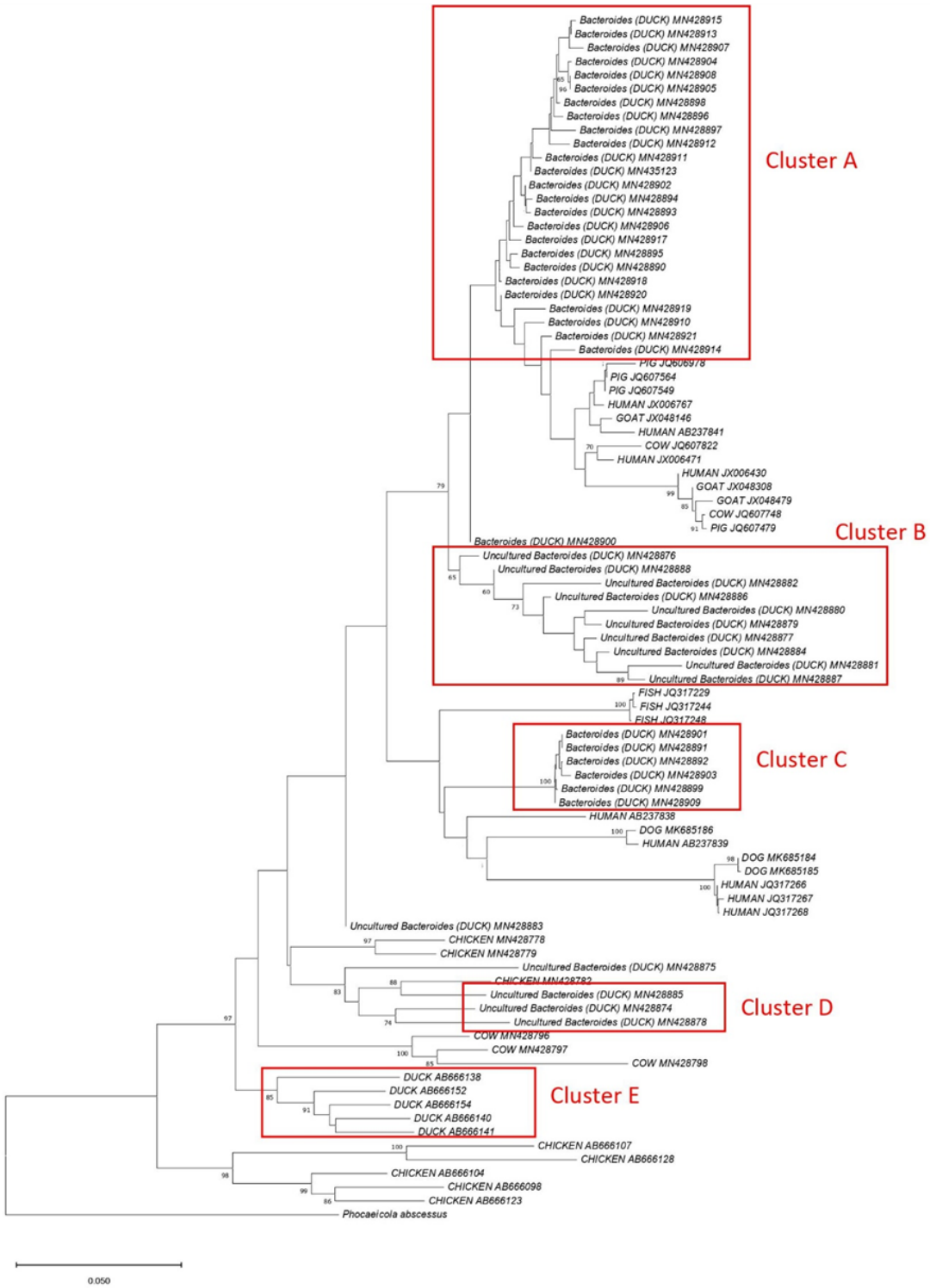

Figure 4 Phylogenetic relationships of cultured and uncultured *Bacteroides* sp. using 16S rRNA gene sequences from fecal samples of ducks. *Phocaeicola abscessus* was used as an outgroup. Values at the nodes are bootstrap values with 1000X resampling analysis. Only values >60% are shown.

In terms of duck DNA sequences, *Bacteroides* grouped into 5 clusters (A, B, C, D, E). Of the 5 clusters, 4 (A–D) were samples from this study, while cluster E was composed of sequences taken from the study of **Kobayashi *et al.* (2013)** that were downloaded from the GenBank. Cluster E composed of gene sequences used in developing duck-associated MST primers in Japan. Within the 4 clusters, clusters A and C came from *Bacteroides* cultures and clusters B and D were sequences from uncultured *Bacteroides* duck fecal samples. Cluster C has a bootstrap support value of 100 followed by cluster E with 85 and cluster D with 83. Cluster B has a bootstrap of 65. Although cluster A contains most of the *Bacteroides* spp., it shows a bootstrap value lower than 60%, hence not shown. Interestingly, all samples from the Philippines clustered and none with cluster E.

DISCUSSION

Worldwide, the duck industry has grown over the years. Similar to chickens, ducks can be consumed for their meat and eggs **(Adzitey and Adzitey, 2011; Rajput *et al.*, 2014; Ismoyowoti and Sumarmono, 2019)**. In the Philippines, there are three known species of ducks. One is the native and endangered Philippine duck (locally called *pato*; *Anas luzonica* L.), while the other two are the commercially marketed non-native ducks, the Muscovy ducks (locally called *itik*; *Cairina moschata* L.) and the Mallard ducks (locally called *bibe*; *Anas platyrhynchos* L.) **(Chang and Dagaas, 2004)**. Muscovy ducks are farmed for their meat, while Mallard ducks are grown for their eggs. The increasing market for duck meat and most especially duck eggs has generated 40.3 billion Philippine pesos accounting to 11% of the total value of Philippine agricultural production. Moreover, as of 2018, the production of duck meat and egg has risen to 31.09 thousand metric tons and 45.42 thousand metric tons, respectively. This has generated earnings of 1.68 billion pesos for duck meat and 1.99 billion pesos for duck egg. However, in contrast with other animal husbandries such as chickens, cows, and pigs, Philippine duck farming is dominated by small-scale backyard industry (70%) (**Chang *et al.*, 2003**).

Unfortunately, the problem with such industry is the unregulated or underregulated fecal waste disposal into small bodies of water that may eventually end up in larger bodies of water allowing exposure of the general populace. Some waterfowls cater pathogens such as enterococci, *E. coli*, *Campylobacter* spp., *Salmonella* spp., and *Cryptosporidium* spp. Ducks, for example, have been shown to harbor *E. coli*, enterococci, *Campylobacter* spp., and *Cryptosporidium* spp. with a prevalence of 95%, 100%, 29%, and 1.3%, respectively (**Moriarty *et al.*, 2011**). In the study of **Murphy *et al.* (2005)**, pathogenic microorganisms such as *Streptococcus alactolyticus/ suis/ bovis, E. coli, Staphylococcus warneri, Enterococcus cecorum, Clostrodium perfringens, Neisseria mucosa*, and *Campylobacter jejuni* were isolated from duck feces. On the other hand, *Aeromonas hydrophila, E. coli, Proteus vulgaris, Sarcina maxima, Streptobacillus moniliformis, Staphylococcus aureus, Enterococcus* spp., and *Streptococcus* spp. were isolated from the feces of domestic ducks in Turkey **(Adegunloye and Adejumo, 2014)**. Oocysts of *Cryptosporidium* were detected from duck feces and high fecal coliform levels attributed to the permanent mallard duck population in a beach in Madison, Wisconsin, USA that caused its closure **(Fleming and Fraser, 2001)**. In the study of **Ramirez-Martinez *et al.*, (2018),** using next generation sequencing, duck feces contained sequences from *Herpesviridae, Adenoviridae, Retroviridae*, and *Myoviridae* viral families. These studies increase our understanding of ducks as reservoir of pathogenic microorganisms and provide the basis for studying and better monitoring of the transmission of pathogens from wild animals to humans.

In this study, we detected *Bacteroides* through the amplification of 16S rRNA gene from total DNA extraction and culture method from fecal samples. *Bacteroides* are Gram negative, non-spore-forming, non-motile, and strictly anaerobic bacteria. They were targeted for isolation because they are known to be consistently present in high numbers in gut and fecal microbiota (**Rivera and Rock, 2011)**. Due to their anaerobic nature, they have little capacity for growth outside the gut of their hosts, making them more suitable for MST since they provide a more accurate count in terms of the degree of fecal pollution (**Rivera and Rock, 2011; Kabiri *et al.*, 2016**). Moreover, there is a strong correlation between the presence of *Bacteroides* and the presence of fecal pathogens such as *E. coli*, *Salmonella*, and *Campylobacter* as opposed to detecting the presence of traditional FIB that does not show a similar correlation (**Walters *et al.*, 2007; Ahmed *et al.*, 2016).** Therefore, due to the characteristics of *Bacteroides* spp., a majority of LIM are *Bacteroides*-based and are designed to target specific sequences within the 16S rRNA gene to differentiate the different sources of contamination by either conventional PCR or quantitative PCR (**Hussein *et al.*, 2014**).

In the phylogenetic analysis, clusters A–D came from Philippine duck fecal samples and their respective nodes/branches formed closer groups with one another, while cluster E (Japan sequences) had a branch distinctly separate from the Philippine duck clusters. This shows an intraspecies difference between *Bacteroides* species in the Philippines and in Japan. This difference may depend on geographical location since clusters A–D were composed of samples collected from the Philippines, and cluster E was composed of samples from Japan. This may affect the sensitivity of MST biomarkers developed in Japan and may not be applicable to Philippine environment. Another feasible reason for this grouping is the diet of the ducks. In this study, the Philippine ducks were kept in cages and fed with the same feeds twice a day. By contrast, the *DuckBac* biomarker designed in Japan came from wild ducks that are free to roam and get exposed to a plethora of food that they can consume. Studies revealed that *Bacteroides* spp. have a high level of host-specificity because they adapt to the gut conditions of their hosts and even have the tendency to coevolve with their host species. Moreover, diet produces a strong selective pressure making gut microbiomes distinct between populations **(Bernhard and Field, 2000; Duncan *et al.*, 2003; Turnbaugh *et al.*, 2009; Garcia-Amado *et al.*, 2018**).

To our knowledge, this study is the first documentation of *B. nordii* from ducks or from an avian species since most of the sequences deposited in GenBank are of mammalian origin. Interestingly, *B. nordii* is consistently isolated and detected from humans. It has been shown as part of human abdominal infections, rectal/anal/perirectal abscesses (**Song *et al.*, 2004; Nagy *et al.*, 2011)**. In addition, *B. nordiii* belongs to the *B. fragilis* group which has shown increasing resistance to amoxicillin, cefoxitin, clindamycin, moxifloxacin, imipinem, metronidazole, and tigecycline. There is evidence of extensive resistance gene transfer among *Bacteroides* spp. (**Shoemaker *et al.*, 2001; Nagy *et al.*, 2011**).

The increasing duck farming industry entails the use of more water resource since ducks are naturally adept in wet or aquatic environments. Logically, as more ducks are produced to sustain the industry so does the amount of duck feces that will be released into the environment further contributing to the aquatic fecal contamination. Unfortunately, ducks have been shown to be reservoirs of pathogenic microorganisms. Moreover, studies have shown that *Bacteroides* has developed extensive antibiotic resistance and can horizontally gene transfer (HGT) the genes via plasmids or conjugative elements (**Shoemaker *et al.*, 2001; Coyne *et al.*, 2014; Huddleston, 2014)**. *Bacteroides* has already been shown to have widespread bacterial intraspecies and intrafamily HGT of antibiotic resistance genes (**Coyne *et al.*, 2014).** *Bacteroides* poses no direct hazard when they are outside the host due to their anaerobic nature, however, within the host gut, *Bacteroides* can affect through HGT other bacteria or transient bacteria that can persist outside the body, to become antibiotic resistant.

Thus, this study has laid the groundwork for the need to include ducks in the monitoring of fecal contamination using the *Bacteroides* directly isolated from ducks. The sequences acquired in this study will serve as basis for constructing duck-specific MST biomarkers.

CONCLUSIONS

Ducks have been shown to harbor pathogens that are zoonotic, hence making them suitable for developing MST biomarkers (**Moriarty *et al.*, 2011**). *Bacteroides* spp. are the preferred bacteria for the development of MST biomarkers because of their many advantages over the traditional FIB place (**Savichtcheva and Okabe, 2006; Oun *et al.*, 2017**). Although culture-based procedures take time, they provide better phenotypic characterization of bacterial isolates. Culture-based methods in conjunction with molecular and metagenomic techniques can help in strengthening the NCBI database since bulk of *Bacteroides* information is generated through metagenomics. The combination of methods provides a higher confidence level in the final selection of biomarkers. Afterall, metagenomics still needs the functional verification that only culture-based methods can provide. Metagenomics provides the target while culture-based methods provide the proof (**Guo *et al.*, 2014**). It is recommended that since 16S rRNA gene sequences from ducks are already stored in GenBank, primer design of duck-specific MST biomarkers should be forthcoming. Future studies on antibiotic susceptibility patterns of *Bacteroides* from ducks can also be done. Detection of antibiotic resistance genes and *Bacteroides* virulence genes can also be studied since there is a gap on this field of research on *Bacteroides* in animals.

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