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ORIGINAL ARTICLE

The culture-dependent and culture-independent analysis for determination of bacterial diversity within *Limnatis nilotica* (Clitellata: Hirudinea)

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Abstract
It has been known that a number of bacterial pathogens living in the digestive tract of leeches such as *Aeromonas veronii* and *A. hydrophila* are related to the blood-sucking behavior of certain species. Therefore, it would be important to describe bacterial species located in the digestive tract of blood-sucking leeches. For this reason, we studied the bacterial diversity of the body surface and the internal organs of *Limnatis nilotica* (Savigny, 1822) (Clitellata: Hirudinea) which is one of the most important parasites in domesticated animals and rarely in humans. In accordance with this purpose, the culture-dependent and culture-independent (PCR-DGGE) methods were used. Genomic DNA was extracted from the bacterial isolates cultivated in pure cultures from the body surface and the leech homogenate and, the total DNA was extracted from the leech homogenates for DGGE analysis. Based on the culture-dependent method, 32 isolates were obtained from the body surface and the leech homogenates and the most common isolated bacterium was *Aeromonas* sp. On the other hand, five bacterial species (*Pasteurella* sp. is the most common) were determined using DGGE analysis. These results could help to find some features of *N. nilotica* biology and lead to investigations associated with host-microbe and microbe-microbe interactions for the purpose of controlling *N. nilotica* in the infested waters.

Keywords Bacteria · The Nile leech · DGGE · 16S rDNA

Introduction

Leeches are characterized by a small sucker, which contains the mouth, at the anterior end of the body and a large sucker located at the posterior end. They can be found in various environments such as freshwater, marine and terrestrial ecosystems (Chandra 1991; Siddall and Burreson 1998). These organisms could be also used as environmental stress indicators in water ecosystems (Klemm 1991). The leeches are located in the class Hirudinea belonging to the phylum Annelida. The class Hirudinea is divided into two orders named as Rhyncbobdellae (leeches with a proboscis) and Arhynchobdellae (leeches without a proposcis) (Chandra 1991). The suborder Hirudiniformes belongs to the order Arhynchobdellae and includes jawed leeches. The jawed leeches usually feed on blood and can be found in both terrestrial and aquatic environments (Seo et al. 2013). Some species of blood-feeding leeches in the families of Glossiphoniidae, Hirudinidae and Haemadipsidae are temporary ectoparasites and generally feed on the blood of vertebrates: amphibians, reptiles, waterfowl, fish and mammals including humans (Keim 1993; Thorp and Covich 2010).

Among blood-feeding leeches, the genus *Limnatis* Moquin-Tandon, 1827 includes four species called *L. nilotica* (Savigny, 1822), *L. bacescui* (Manoleli, 1972), *L. paluda* (Tennent, 1859) and *L. haasi* (Johansson, 1927) (Sawyer 1986; Jueg 2008; Nakano et al. 2015). *Limnatis nilotica* (the Nile leech) has a widespread distribution worldwide and can be found in lakes, streams and other freshwaters in southern Europe, Middle East, North Africa, North Africa and Asia (Sawyer 1986). Nesemann and Neubert (1999) presented a simple key for identification of four *Limnatis* species based on morphological features. In addition, based on 12S, 18S, 28S and mitochondrial cytochrome c oxidase subunit I

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(COI) genes, there are some studies showing the phylogenetic position of this genus within the order Arhynchobdellida (Borda and Siddall 2004). Recently, Nakano et al. (2015) showed that two species of this genus (*L. nilotica* and *L. paluda*) could be easily distinguished from each other based on mitochondrial cytochrome c oxidase subunit I (COI) and 12S gene sequences.

It has been reported that *L. nilotica* can feed on the blood of many warm-blooded animals such as cow, sheep, goat and horse and even humans. This leech can not make incision in the skin since its piercing organ is too weak to cut the skin. Therefore, it usually causes main problems in mucous membranes of the pharynx, larynx and nostrils by attacking animals and humans. It can rarely invade the eye, urethra or vagina (Orevi et al. 2000). It has been also reported that some leeches belonging to *Hirudo* spp. may transmit pathogenic microorganisms such as *Aeromonas* sp. to their host (Nelson and Graf 2012). Therefore, it is important to identify bacterial endosymbionts of this leech to understand the role of these symbionts in the biology of *L. nilotica* and to develop possible control methods in the infested waters. Although *L. nilotica* has been parasitizing many pets, farm animals and humans, there are no studies to determine its bacterial endosymbionts.

Various microorganisms such as bacteria and yeasts in the intestinal systems of animals and humans play an important role in the health and nutrition. Like many animals, leeches have their own microbial symbionts in their digestive tracts (Nelson and Graf 2012). Up to now, many researchers investigated the internal bacterial diversity of various leech species using the culture-dependent and culture-independent techniques (Nomomura et al. 1996; Kikuchi and Fakatsu 2002; Goffredi et al. 2012). There are a few hypotheses about the roles of these microorganisms in the various internal parts of blood-feeding leeches. The first one is that the microbial symbionts may be responsible for digestion of the blood. The second one is that the microbial symbionts can synthesize essential nutrients which the leech can't produce for itself. The third one is that the microbial symbionts can provide a resistance to prevent the colonization of pathogenic or detrimental bacteria (Nelson and Graf 2012). Therefore, it is crucial to identify bacterial species located in the digestive tract of blood-feeding leeches. This can provide to understanding the roles of these bacterial species in the leech biology.

The determination of microbiota can be performed using different techniques such as culture-dependent and culture-independent methods. Based on the culture-dependent method, bacteria are cultured in pure and then, characterization studies are performed. However, according to our current knowledge, we know that almost 99% of microbes can not be cultivated (Graf et al. 2006). Therefore, the culture-independent techniques such as PCR-denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), the direct cloning of environmental DNA, restriction fragment length polymorphisms (RFLP), terminal restriction fragment length polymorphisms (T-RFLP), quantitative PCR (qPCR), fluorescent in situ hybridization (FISH), microarray and next generation sequencing can provide much more reliable, fast and economical aspects for determination of bacterial diversity (Graf et al. 2006; Su et al. 2012; Adewumi et al. 2013; Deutscher et al. 2018). Therefore, culture-independent methods seem to be ideal for identifying bacterial species within leeches since a small number of bacteria are likely to be cultivable.

In this study, we aimed to determine the bacterial diversity within the Nile leech using culture-dependent and culture-independent techniques (PCR-DGGE) for expanding our knowledge on the occurrence of bacteria within blood-feeding leeches. The obtained data from can be helpful to understand the role of bacterial species in biology of the Nile leech and to control this leech in the infested waters. This is the first study to determine the internal bacterial diversity of *L. nilotica*.

Materials and methods

Collection of leech samples

Leech samples were collected from different artificial pools in the same locality of Kırşehir, Turkey during the spring of 2017. Leeches were collected by an aquarium scoop. After that, the collected leeches were put into a plastic bottle with the appropriate amount of pool water and were brought to the laboratory for bacterial isolation and DNA extraction.

Identification of the leech

Firstly, the collected leeches were morphologically identified. Leeches with the following characteristics were identified as *L. nilotica*; two different color patterns on the dorsal side (the typical pattern is green to brown with four interrupted black lines, while some specimens have an additional median orange band), marginal two orange stripes, dark brown ventral side, five pairs of parabolic eyes on the anterior sucker, very large posterior sucker and the genital pores which are separated by five annuli (Nesemann and Neubert 1999; Bahmani et al. 2012; Ahmed et al. 2015).

The collected leeches were also molecularly identified using gene sequencing. DNA extraction was performed from the caudal sucker tissue in order to prevent a possible contamination from internal regions. DNA extraction was performed using the DNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. After DNA extraction, approximately 650 bp fragment of mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified the following primer pairs; LCO1490 (5′- GGTCAACAAATCAT

AAAGATATTGG-3′) and HCO2198 (5’-TAAACTTC AGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994; Borda and Siddall 2004). PCR conditions were adjusted according to the study of Borda and Siddall (2004). 10 μl of the PCR product was analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide to check the size of the amplicon. Finally, the correct PCR product was sent to MACROGEN (The Netherlands) for sequencing. The sequencing reaction was performed by the amplification primers for both strands. The obtained sequence was subjected to the nucleotide BLAST searches in the NCBI GenBank database and they were used for phylogenetic analysis for further identification studies (Altschul et al. 1990).

Isolation and cultivation of bacteria

The collected leeches were kept alive until the bacterial isolation was done. Firstly, body surfaces of the leeches were sampled by a sterile swap and spread on Trypticase Soy Agar (BBL) supplemented with sheep blood 5%. Secondly, the leech samples were dipped into 80% ethanol for 10 s to prevent a possible contamination from surface microorganisms. After that, they were aseptically removed from ethanol and washed three times with sterile distilled water. Then, the leeches were dissected with a sterile lancet and all internal organs were removed into a sterile glass tube (10 ml). All of the internal organs were homogenized by a sterile tissue grinder, pooled and used for bacterial isolation. A total of 10 leeches were used for homogenization. After homogenization, serial dilutions from 10^{-1} to 10^{-8} were prepared and each dilution was spread on Trypticase Soy Agar (BBL) supplemented with sheep blood 5%. The plates were incubated at 37 °C for 2 days in dark. At the end of the incubation period, single colonies were selected and transferred to another blood agar to obtain pure cultures. The obtained pure cultures were stocked in 20% glycerol at −20 °C for further studies.

16S rRNA gene sequencing of the cultivated bacteria

The total genomic DNA was extracted from the isolated bacteria using the standard phenol/chloroform procedures (Sambrook et al. 1989). Approximately a 1500 bp fragment of the 16S rRNA gene was amplified using the primer pairs of 27F (5′-AGAGTTTGATCMTGGCTCAG-3′ as forward) and 1492R (5′-GGYTACCTTGTTACGACTT-3′ as reverse). Primers were purchased from Macrogen (The Netherlands). PCR conditions were adjusted according to the study of Demirci et al. (2013). The amplified PCR products were sequenced by the primer pairs of SP6 and T7 in Macrogen. The obtained sequences were used to carry out BLAST searches using NCBI GenBank database to find out percent similarities of the bacterial isolates with their closely related bacterial species (Altschul et al. 1990). In addition, the sequences were used to construct a pyhlogenetic tree.

PCR-denaturing gradient gel electrophoresis (DGGE)

PCR-DGGE method was used to identify unculturable bacteria within *L. nilotica*. The collected leech samples were firstly surfaced sterilized by 80% ethanol and dissected using a sterile lancet to remove all of the internal organs. The removed internal organs from 10 leech specimens were homogenized and used for the total DNA extraction. The total DNA extraction was performed using Qiagen DNeasy Blood and Tissue kit (QIAGEN, Valencia, CA) according to the manufacturer’s protocol. Firstly, the extracted total DNAs were used as template to amplify the V3 region of 16S rRNA genes. For this amplification, the primer pairs of (5′-CGCCCGCCGCGCGC GGCGGGCGGGGCGGGGGCACGGGGGGCCTA CGGGAGGCAGCAG-3′ as forward) and (5′-ATTA CCGCGGCTGCTGG-3′ as reverse) were used. The 5′ end of the forward primer contained a 40 base-pair GC-clamp. This clamp is important for downstream DGGE applications to keep DNA fragments stable (Andrews 2013). PCR conditions were adjusted according to the protocol of Andrews (2013) which was originally adapted for DGGE analysis from arthropods. The thermal cycler program included touchdown PCR which is a special PCR protocol increasing both specificity and yield of the final product. After amplification, the PCR products were separated on 1.5% agarose gel containing ethidium bromide and visualized under UV light. Approximately 150 bp PCR products were obtained and used for DGGE analysis.

Denaturing gradient gel electrophoresis (DGGE) of the PCR products was performed using DGGE package system purchased from Cleaver Scientific (Warwickshire, UK). The amplified PCR products were purified using QIAquick gel extraction kit (Qiagen, Valencia, CA) and all PCR products containing 5 μl loading dye were loaded into wells of 10% (*w/v*) polyacrylamide gel. Before loading of PCR products, the buffer was pre-heated to 60 °C. The optimal separation was achieved with a 30–70% urea-formamide denaturing gradient. Gel electrophoresis was performed at 60 °C, 130 V for 16 h. Once the electrophoresis was completed, the gel was removed from the glass plates and transferred on a staining box and stained with ethidium bromide for 40 min and visualized under UV light. After detection of bands, each band was excised from the gel using a sterile spatula and transferred into microcentrifuge tube. After that, the PCR products located within the gel were extracted according to the protocol of Andrews (2013). 2 μl of the extracted DNA was amplified using the same primers mentioned above and the amplified products were confirmed by electrophoresis. Finally, these amplified PCR products were cloned into pJET1.2 vector system (Thermo Fisher Scientific Inc., USA) and sequenced. The

obtained sequences were used to carry out BLAST searches using NCBI GenBank database and they were used for phylogenetic tree construction (Altschul et al. 1990).

Phylogeny

All obtained sequences were subjected to phylogenetic analysis to ascertain the exact species identification. The sequences were edited by Bioedit 7.1.3.0 software. Multiple sequence alignments were performed with ClustalW packed in Bioedit 7.1.3.0 (Hall 1999). Finally, the sequences were subjected to neighbor-joining analysis with p-distance correction, gap omission and 1000 bootstrap pseudoreplicates using MEGA 6.0 (Tamura et al. 2013).

GenBank accession numbers

Accessions number of the mitochondrial cytochrome c oxidase subunit I (COI) gene for *L. nilotica* is MG831390. Accession numbers for the 16S rRNA genes belonging to the cultivated and uncultivated isolates were provided in Table 1 and Table 2, respectively. All gene sequences were deposited in NCBI GenBank database.

Results

Identification of the leech

The collected leech specimens were morphologically identified as *Limnatis nilotica* (Savigny, 1822) based on certain characteristics as mentioned above. Separately, leech specimens were molecularly identified based on the partial sequence of mitochondrial cytochrome *c* oxidase subunit I (COI). The leech specimens used in this study were compared with reference species which were indicated in the study of Borda and Siddall (2004) using phylogenetic analysis. It was shown that our species was identical to *L. nilotica* based on the phylogram (Fig. 1).

Isolation and identification of the cultivated bacteria

A total of 32 bacterial isolates were obtained from the body surface and leech homogenates (19 of them were from homogenates) and all of them were cultivated in pure cultures and stocked at −20 °C in the lab of Genetic and Bioengineering, Ahi Evran University. All cultivated bacteria were identified based on 16S rRNA gene sequencing. Among 32 isolates, 21 isolates were detected to be in the phylum Proteobacteria. Within these bacteria, 12 isolates belonged to the genus *Aeromonas* with four species: *A. hydrophilia* (EEA-1), *A. allosaccharophila* (EEA-5) and *A. veronii* (EEA-13, EEA-15 and EEA-32 and *Aeromonas* sp. (7 isolates). While three isolates (EEA-6, EEA-12 and EEA-26) were identified as *Shewanella putrefaciens*, another three isolates (EEA-8, EEA-16 and EEA-27) were identified as *Shewanella* sp. The isolates of EEA-21, EEA-23 and EEA-25 were identified as *Pseudomonas* sp., *Ralstonia picketti* and *Massilia* sp., respectively.

Within the phylum Firmicutes, nine bacteria were identified. Four strains (EEA-4, EEA-14, EEA-19 and EEA-22) were identified as *Staphylococcus* sp. One isolate (EEA-31) was identified as *Bacillus* sp. The remaining four isolates (EEA-30, EEA-28, EEA-20 and EEA-29) were identified as *Staphylococcus simulans, Staphylococcus hominis, Staphylococcus epidermidis* and *Bacillus pumilus*, respectively.

Within the phylum Bacteroidetes, only two isolates (EEA-3 and EEA-24) were identified as *Chryseobacterium* sp. The detailed information about the cultivated bacterial strains was given on Table 1. All species identifications were confirmed by phylogenetic analysis (Fig. 2).

Bacterial identification based on DGGE

A total of 8 bands were determined on the DGGE gel. The determined bands were excised from the gel and kept in 100 µl sterile dH_2O overnight. On the following day, the supernatants were amplified by PCR, cloned into pJET1.2 vector system (Thermo Fisher Scientific Inc., USA) and sent to Macrogen for sequencing. Random sequencing of different clones identified the presence of seven uncultured Proteobacteria (*Pasteurella canis* (EEA-33 and EEA-35), *Pasteurella* sp. (EEA-34, EEA-37 and EEA-38), *Mannheimia varigena* (EEA-39), *Actinobacillus* sp. (EEA-40)) and one uncultured Firmicutes (*Streptococcus dysgalactiae* (EEA-36) (Table 2, Fig. 3).

Discussion

The genus of *Aeromonas* includes gram-negative rods which are widely distributed in freshwater and marine environments (Holmes et al. 1996). Some species belonging to this genus such as *A. hydrophila*, *A. luqiefaciens*, *A. caviae* and *Aeromonas sobria* are known as causative agents of a wide spectrum of diseases in man and animals. Humans can be infected with these bacteria from different environmental sources (Janda and Abbott 2010). It has been also shown that some blood-feeding leeches such as *Hirudo medicinalis* (Linneaus, 1758), *H. verbana* (Carena, 1820), *H. orientalis* (Utevsky & Trontelj, 2005) and *Macrobdella decora* (Say, 1824) harbor different *Aeromonas* species (especially *A. hydrophila* and *A. veronii*) which may cause infections in humans (Nelson and Graf 2012). Especially, *Aeromonas* species is the most common cause of infections after the use of

Table 1 The suggested identifications of the cultivated bacterial isolates from *L. nilotica* with their GenBank accession numbers and their closest relatives based on NCBI blast search (Altschul et al. 1990)

Phylum	Strain number	Suggested identification	The closest relative in GenBank and their accession numbers	Percent (%) identity	Source	GenBank accession number
Proteobacteria	EEA-1	*Aeromonas hydrophila*	*Aeromonas hydrophila* (AY827493)	99	Homogenate	MG822754
	EEA-5	*Aeromonas allosaccharophila*	*Aeromonas allosaccharophila* S5–33 (KC202276)	99	Body surface	MG822758
	EEA-2	*Aeromonas* sp.	*Aeromonas salmonicida* subsp. *pectinolytica* (CP022426)	94	Body surface	MG822755
	EEA-7		*Aeromonas* sp. J1.3E2 (KF317749)	99	Body surface	MG822760
	EEA-9		*Aeromonas* sp. J1.3E2 (KF317749)	98	Homogenate	MG822762
	EEA-10		*Aeromonas* sp. J1.3E2 (KF317749)	99	Homogenate	MG822763
	EEA-11		*Aeromonas* sp. J1.3E2 (KF317749)	99	Homogenate	MG822764
	EEA-17		*Aeromonas* sp. J1.3E2 (KF317749)	99	Homogenate	MG822770
	EEA-18		*Aeromonas* sp. J1.3E2 (KF317749)	99	Homogenate	MG822771
	EEA-13	*Aeromonas veronii*	*Aeromonas veronii* NX16104 (KY767507)	99	Homogenate	MG822766
	EEA-15		*Aeromonas veronii* AVZ01 (MF521598)	99	Homogenate	MG822768
	EEA-32		*Aeromonas veronii* NX16104 (KY767507)	99	Homogenate	MG822785
	EEA-6	*Shewanella putrefaciens*	*Shewanella* sp. S5–28 (KC202274)	99	Body surface	MG822759
	EEA-12		*Shewanella* sp. S02 (FJ002583)	99	Homogenate	MG822765
	EEA-26		*Shewanella putrefaciens* (AB681550)	99	Body surface	MG822779
	EEA-8	*Shewanella* sp.	*Shewanella* sp. S02 (FJ002583)	99	Homogenate	MG822761
	EEA-16		*Shewanella* sp. S5–28 (KC202274)	99	Homogenate	MG822769
	EEA-27		Uncultured *Shewanella* sp. LHN64 (KF003193)	99	Homogenate	MG822780
	EEA-21	*Pseudomonas* sp.	*Pseudomonas fluorescens* G100814 (HQ874650)	99	Body surface	MG822774
	EEA-23	*Ralstonia picketti*	*Ralstonia pickettii* 149 (EU730922)	99	Homogenate	MG822776
	EEA-25	*Massilia* sp.	*Massilia* sp. 51Ha (FR865961)	99	Body surface	MG822778
Firmicutes	EEA-31	*Bacillus* sp.	*Bacillus mojavensis* PbT6 (KT717630)	98	Body surface	MG822784
	EEA-29	*Bacillus pumilus*	*Bacillus pumilus* XJAS-ZB-14 (FJ237280)	98	Body surface	MG822782
	EEA-30	*Staphylococcus simulans*	*Staphylococcus simulans* FDAARGOS_383 (CP023497)	99	Body surface	MG822783
	EEA-28	*Staphylococcus hominis*	*Staphylococcus hominis* K23 (KU922442)	99	Homogenate	MG822781
	EEA-20	*Staphylococcus epidermidis*	*Staphylococcus epidermidis* (AB617572)	99	Homogenate	MG822773
	EEA-4	*Staphylococcus* sp.	*Staphylococcus* sp. VITS-3 (EU807751)	99	Body surface	MG822757
	EEA-14		*Staphylococcus* sp. dv8 (FJ773995)	99	Homogenate	MG822767
	EEA-19		*Staphylococcus* sp. MRSE4 (KF048925)	99	Homogenate	MG822772
	EEA-22		*Staphylococcus* sp. MRSE4 (KF048925)	97	Homogenate	MG822775
Bacteroidetes	EEA-3	*Chryseobacterium sp.*	*Chryseobacterium* sp. TH1 (JN208181)	99	Body surface	MG822756
	EEA-24		*Chryseobacterium* sp. TH1 (JN208181)	98	Body surface	MG822777

medicinal leech therapy for many clinical pictures. Although the soft-tissue infection of *Aeromonas* species can be seen at the rate of 7 to 20%, *Aeromonas* wound infection is very rare following leech bites in the wild (Clark et al. 2001). Although it is known that some *Aeromonas* species can cause infections in warm-blooded and cold-blooded animals including fish, reptiles, amphibians and mammals, there is no report about *Aeromonas* infections in any animals following the leech bite. In this study, we isolated 12 *Aeromonas* strains from the Nile leech including different species such as *A. hydrophila*, *A. veronii*, *A. allosaccharophila* and *Aeromonas* sp. These *Aeromonas* species are most likely responsible for the digestion of blood meal in the leech intestine. According to the literature, it might also be possible that the *Aeromonas* species isolated in this study may be transmitted to the host animals or humans after biting and may cause infections.

The genus of *Shewanella* (family of Vibrionaceae) includes bacterial species which are saprophytic gram-

Table 2 The suggested identifications of the uncultivated bacterial isolates from *L. nilotica* with their GenBank accession numbers and their closest relatives based on NCBI blast search (Altschul et al. 1990)

Phylum	Strain number	Suggested identification	The closest relative in GenBank and their accession numbers	Percent (%) identity	Source	GenBank accession number
Proteobacteria	EEA-33	*Pasteurella canis*	*Pasteurella canis* CLR2014–1 (KM079615)	100	Homogenate	MH005076
	EEA-34	*Pasteurella* sp.	*Pasteurella dagmatis* NCTC11617 (LT906448)	100	Homogenate	MH005077
	EEA-35	*Pasteurella canis*	*Pasteurella canis* CLR2014–1 (KM079615)	100	Homogenate	MH005078
	EEA-37	*Pasteurella* sp.	*Pasteurella dagmatis* NCTC11617 (LT906448)	100	Homogenate	MH005080
	EEA-38	*Pasteurella* sp.	*Pasteurella dagmatis* NCTC11617 (LT906448)	100	Homogenate	MH005081
	EEA-39	*Mannheimia varigena*	*Mannheimia varigena* USDA-ARS-USMARC-1388 (CP006953)	100	Homogenate	MH005082
	EEA-40	*Actinobacillus* sp.	*Actinobacillus* sp. NE-151 (AB493823)	99	Homogenate	MH005083
Firmicutes	EEA-36	*Streptococcus dysgalactiae*	*Streptococcus dysgalactiae* subsp. *dysgalactiae* (KY118916)	99	Homogenate	MH005079

negative rods. The members of this genus are widely distributed in several environments from soil to aquatic reservoirs (Holt et al. 2005). It has been known that they are initially colonized on previously damaged tissues and cause infections (Winn et al. 2006). Some species (*S. putrefaciens*, *S. algae*) within this genus are known to be rarely pathogenic in fish and human (Vignier et al. 2013; Pazdzior 2016). Schulz and Faisal (2010) showed that *S. woodyi* is associated with the leech *Myzobdella lugubris* (Leidy, 1851). Based on the literature, it is possible to say that many healthy freshwater or marine animals may be contaminated with *Shewanella* species. In this study, we isolated six *Shewanella* sp. (one of them is *S. putrefaciens*) from *L. nilotica*. It is seen that the

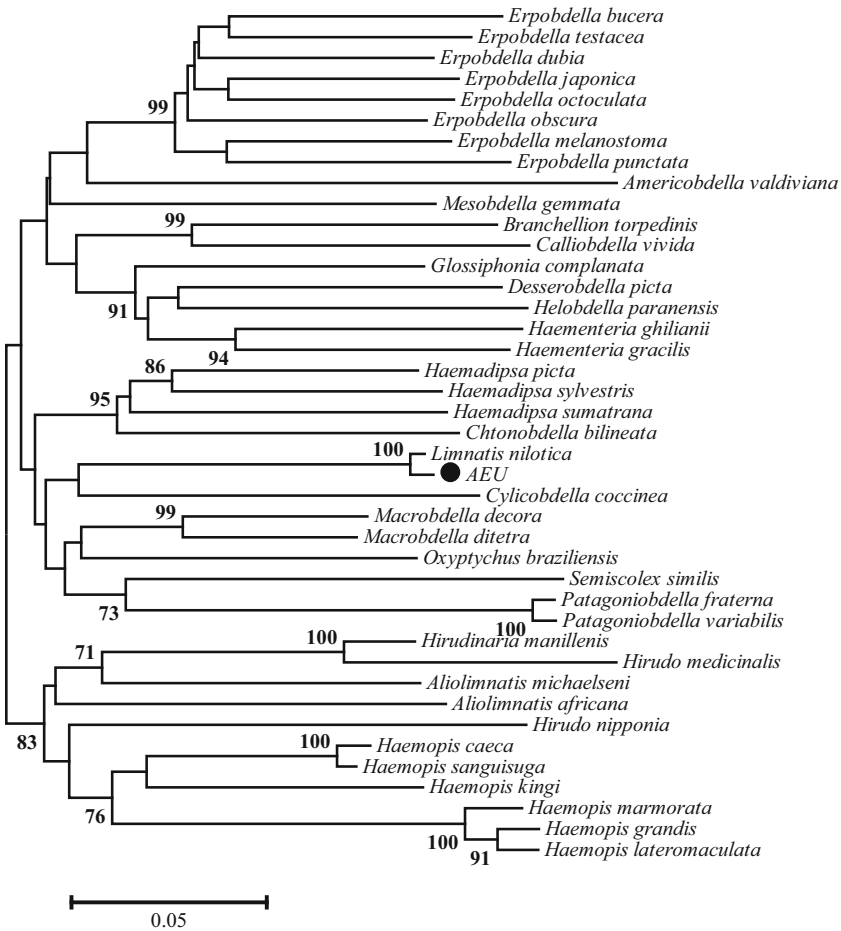

Fig. 1 Phylogenetic tree constructed from neighbor-joining analysis in MEGA 6.0 using the partial sequences of mitochondrial cytochrome *c* oxidase subunit I (COI) belonging to our leech and reference leeches used in the study of Borda and Siddall (2004). Bootstrap values based on 1000 replicates were indicated above nodes. Bootstrap values C ≥ 70 are labeled. The leech used in this study was indicated with black circle. The scale on the bottom of the phylogram indicates the degree of dissimilarity

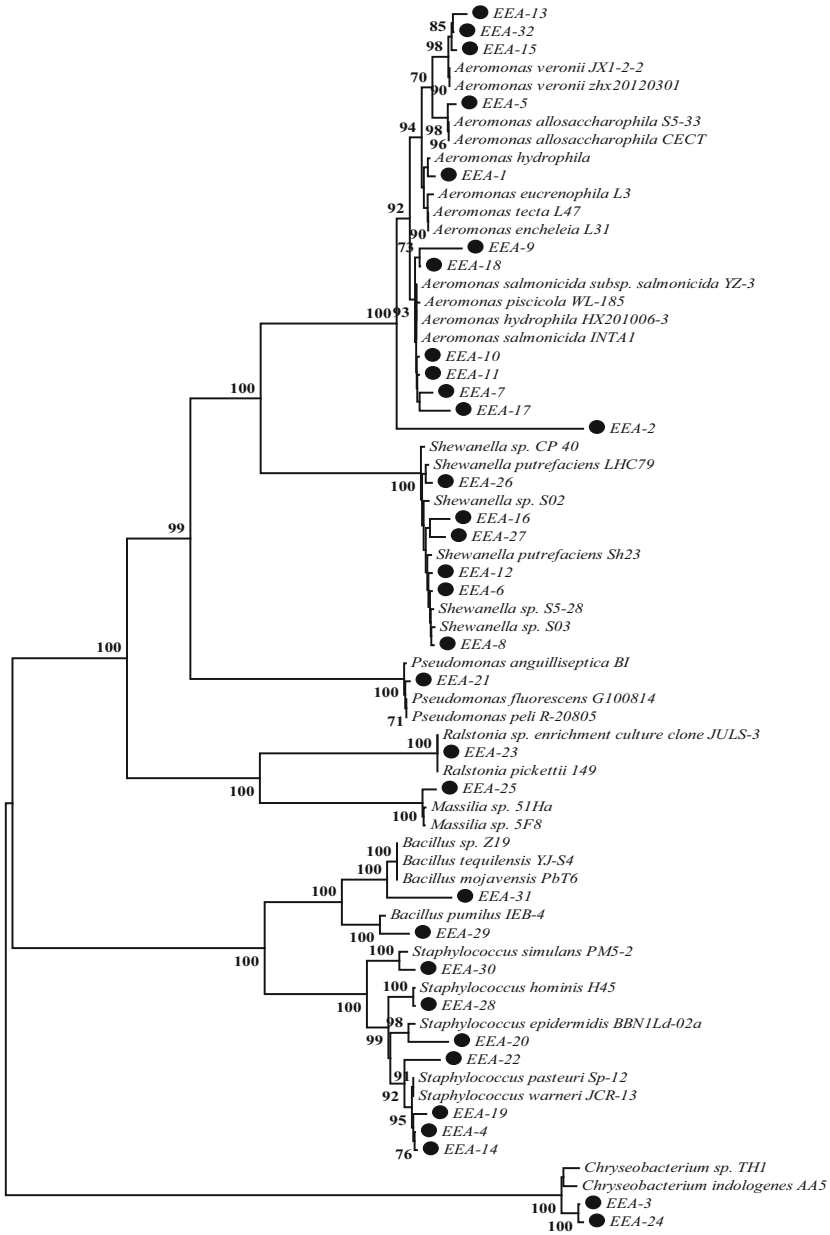

Fig. 2 Phylogenetic tree constructed from neighbor-joining analysis in MEGA 6.0 using 16S rRNA gene sequences belonging to the cultivated bacteria in this study and their closely related bacterial species taken from NCBI GenBank. Bootstrap values based on 1000 replicates were indicated above nodes. Bootstrap values C ≥ 70 are labeled. The cultivated bacteria in this study were indicated with black circle. The scale on the bottom of the phylogram indicates the degree of dissimilarity

members of this genus seem to be closely related to *N. nilotica* but they are probably not pathogenic to the parasitized animals.

In this study, some other bacterial species such as *Pseudomonas* sp. and *Chryseobacterium* (gram negative rods) were also isolated from *L. nilotica*. These bacteria were previously isolated from different leeches such as *H. medicinalis* and *H. verbena* (Nomomura et al. 1996; Worthen et al. 2006). In addition, to our knowledge, we here report that *Ralstonia picketti* and *Massilia* sp. were first time isolated from any leech species. As for gram positive bacteria, two *Bacillus* sp. (one of them is *B. pumilus*) and seven *Staphylococcus* sp. (three of them are *S. simulans*, *S. hominis* and *S. epidermidis*) were isolated from *L. nilotica*. Although there is evidence showing the isolation of *Staphyloccocus* species from leeches (Nomomura et al. 1996), this study showed the first isolation of *Bacillus* species from any leech. Determination of the roles of these bacteria within *N. nilotica* biology needs further investigations.

We defined eight bands on the DGGE gel in order to determine the uncultured bacterial diversity within *L. nilotica*. The variable region 3 (V3) of the bacterial 16S rRNA gene was successfully employed as previously reported for determination of several microbial communities (He et al. 2013; Tagliavia et al. 2014). In the present study, the use of DGGE method resulted in a lower bacterial diversity and showed different bacterial species in comparison to the culture-dependent method. There may be several reasons for these

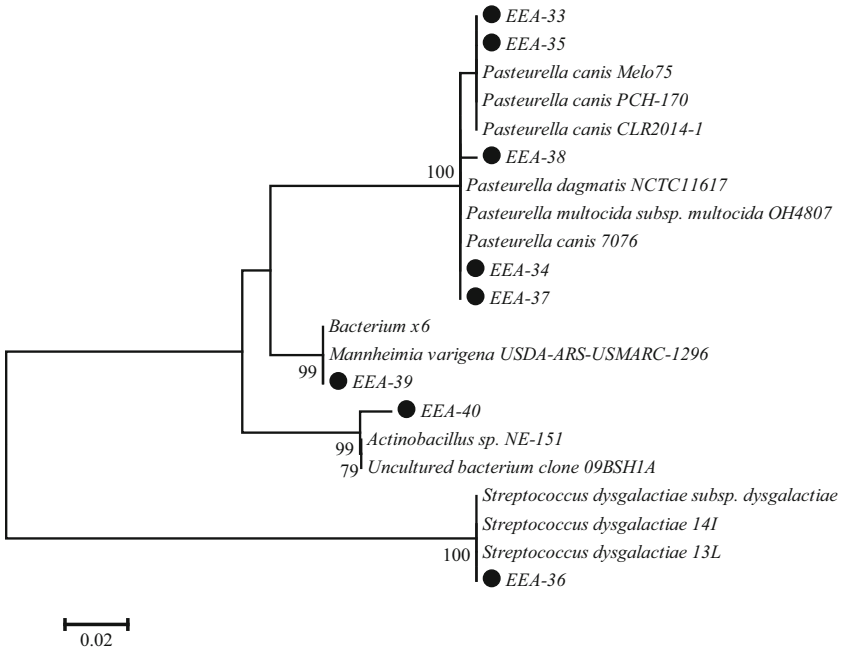

Fig. 3 Phylogenetic tree constructed from neighbor-joining analysis in MEGA 6.0 using 16S rRNA gene sequences belonging to the uncultivated bacteria in this study and their closely related bacterial species taken from NCBI GenBank. Bootstrap values based on 1000 replicates were indicated above nodes. Bootstrap values C ≥ 70 are labeled. The cultivated bacteria in this study were indicated with black circle. The scale on the bottom of the phylogram indicates the degree of dissimilarity

differences. For example, some of the bacteria in low amounts from the homogenate samples can grow rapidly, easily cultured and detected on agar media. However, some bacteria in high amounts couldn't be identified since the artificial culture conditions can not be suitable for their growth. Therefore, both techniques (culture-dependent and culture-independent) should be used for the purpose of determination of bacterial communities in several environments.

Based on the DGGE profiles, the most common detected bacterium is *Pasteurella* sp. which was represented by five bands. Members of this genus are usually considered as opportunistic and secondary invaders in many vertebrates (Christensen and Bisgaard 2006). Within this genus, *P. canis* (represented by two bands in this study), *P. multocida*, *P. dagmatis* and *P. stomatis* are known to cause zoonotic infections in humans after animal bite (Jorgenesen and Pfaller 2015). Therefore, *L. nilotica* might cause *Pasteurella* infections in parasitized animals or rarely humans after the leech bite. The other determined bacteria (*Mannheimia varigena*, *Actinobacillus* and *Streptococcus dysgalactiae*) on the DGGE gel are reported to cause various infections in animals (Rcyroft and Garside 2000; Catry et al. 2004; Abdelsalam et al. 2013).

Conclusions

We described culturable and unculturable bacterial species from *L. nilotica* which is one of the most important parasites of many domestic animals and rarely humans (Orevi et al. 2000). The most common determined bacteria are *Aeromonas* sp. and *Pasteurella* sp. Further studies are needed to find the roles of these bacteria in *L. nilotica* biology. Transmission of zoonotic pathogens by *L. nilotica* should be also studied to prove disease transmission from the leech to animals.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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