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Multilocus sequence analysis revealed a high genotypic diversity of *Aeromonas hydrophila* infecting fish in Uganda

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Abstract

A multilocus sequence analysis (MLSA) was carried out to delineate *Aeromonas hydrophila* from fish in Uganda. Five housekeeping genes including *recA*, *gyrB*, *metG*, *gltA* and *pps;* and the 16S *rRNA* gene were amplified and sequenced from a total of nine *A. hydrophila* isolates. The obtained sequences were edited, and consensus sequences generated for each gene locus. The housekeeping gene sequences were concatenated and phylogenetic analysis performed in MEGA version 7.0.2. Pairwise distances ranged from 0.000 to 0.118, highest within the *gltA* gene locus and lowest within the 16S *rRNA* gene. The average evolutionary diversity within isolates from the same source ranged between 0.002 and 0.037, and it was 0.033 between the different sources. Similar tree topologies were obtained from the different gene loci with *recA*, *metG* and *gyrB* being more consistent in discriminating isolates according to sources while the 16S rRNA gene had the lowest resolution. The concatenated tree had the highest discriminatory power. This study revealed that *A. hydrophila* strains infecting fish in Uganda are of diverse genotypes suggesting different sources of infection in a given outbreak. Efforts to minimize spread of the bacteria across sources should be emphasized to control infections of mixed genotypes.

KEYWORDS
Aeromonas hydrophila, fish, genotypic diversity, multilocus sequence analysis, Uganda

1 | INTRODUCTION

Aeromonas is a genus of Gram-negative bacteria belonging to family Aeromonadaceae (Colwell, MacDonell, & DeLey, 1986). The species are ubiquitous in terrestrial and aquatic environments, with over 19 recognized species (Beaz-Hidalgo, Alperi, Figueras, & Romalde, 2009) infecting a wide range of hosts including humans and fish (M. L. Janda & Abbott, 2010; Palu et al., 2006). Depending on the immunity of the host, the infection may result into clinically important disease. Of great importance in fish production is *A. hydrophila*, one of the major causative agents of motile *Aeromonas* septicaemia, a deadly disease affecting several species of cultured and wild fish across the world (Rasmussen-Ivey et al., 2016). Various approaches have been used to characterize bacteria, and use of genomic data provides a more clear understanding of their evolutionary, pathogenic and epidemiological characteristics (Jolley & Maiden, 2010).

Several studies have aimed at understanding the phylogenetic relationship of genus *Aeromonas;* however, taxonomy is still a challenge due to the complexity of this genus with continuous additions of newly described species (Beaz-Hidalgo et al., 2009; M. L. Janda & Abbott, 2010; Martino, Fasolato, Montemurro, Novelli, & Cardazzo, 2014). A number of genotyping techniques have been used to

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characterize *Aeromonas* species isolated from different sources worldwide with varying success. Such techniques include DNA-DNA hybridization, 16S *rRNA* gene ribotyping, randomly amplified polymorphic DNA (RAPD) PCR, amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE) and multiplex PCR. These techniques, however, have a number of disadvantages including low discriminatory power, poor reproducibility, data portability and they are laborious (A. J. Martinez-Murcia, Benlloch, & Collins, 1992; Sen, 2005), which limit their applications. Some of these techniques use a single gene, commonly the 16S *rRNA* gene (Case et al., 2007), a gene characterized by limited intragenomic heterogeneity in some bacteria that results in failure to resolve different isolates (Morandi, Zhaxybayeva, Gogarten, & Graf, 2005).

Discrimination of the *A. hydrophila* complex has been a major challenge. In this group of bacteria, the 16S *rRNA* gene is highly conserved and it cannot distinguish between some species (Martínez-Murcia et al., 2005). In addition, this gene has multiple heterogeneous copies within the genome of some bacteria including *Aeromonas* spp. (Dahllöf, Baillie, & Kjelleberg, 2000; Fogel, Collins, Li, & Brunk, 1999) which further limit its application in phylogenetic studies. Commonly, it may be separate point mutations in only a single or a few of the 16S *rRNA* gene copies in the same genome. Although sequence analyses based on some single genes such as the *rpoB*, *recA* and *gyrB* have been reported to be excellent molecular markers for phylogenetic inference in the genus *Aeromonas* (Lamy, Laurent, & Kodjo, 2010; Sepe, Barbieri, Peduzzi, & Demarta, 2008; Yáñez, Catalán, Apráiz, Figueras, & Martínez-Murcia, 2003), single genes may not indicate the true phylogenetic relationships (Glaeser & Kämpfer, 2015) as this reflects a very small portion of the genome. Use of several genetic markers has eliminated the bias arising from use of single genes in phylogenetic analyses (Glaeser & Kämpfer, 2015). More reliable tools for identification of bacteria species under genus *Aeromonas* such as the *Aeromonas* MLSTyper that identifies *Aeromonas* species by multilocus sequence typing (MLST) have recently been described (http://196.1.114.46:1800/aeromonas/RTD.html).

Multilocus sequence analysis (MLSA), also known as multilocus phylogenetic analysis (MLPA) (Alperi, Martínez-Murcia, Ko, et al., 2010; Alperi, Martínez-Murcia, Monera, Saavedra, & Figueras, 2010; Figueras et al., 2011), characterizes closely related bacterial isolates by typing several gene loci using the DNA sequences of internal fragments of housekeeping genes. About 450- to 500-bp internal fragments of each gene are used as DNA of this size can be accurately sequenced on both strands using an automated DNA sequencer (Jolley & Maiden, 2010) at a relatively low cost. MLSA has been successfully used to infer bacteria phylogeny and delineation of species (Glaeser & Kämpfer, 2015) including *Aeromonas* spp. (Antonio J. Martinez-Murcia et al., 2011) and other bacteria such as *Mycobacterium* spp (Macheras et al., 2011), *Vibrio* spp (Martens et al., 2008; Sawabe, Kita-Tsukamoto, & Thompson, 2007) and *Edwardsiella tarda* (Abayneh, Colquhoun, & Sørum, 2012). Several genes have provided powerful tools for inter- and intraspecies discrimination of *Aeromonas* spp. including *gyrB*, *recA*, *ppsA*, *metG* and *gltA* (Kupfer, Kuhnert, Korczak, Peduzzi, & Demarta, 2006; Martino et al., 2011). Clear understanding of the aetiological agents of disease and the associated epidemiological parameters is key in planning better treatment and control strategies. Despite the fact that the genus *Aeromonas* is highly prevalent and has been associated with disease in fish in Uganda (Wamala et al., 2018), no detailed studies have been conducted to determine the extent of the problem and the circulating species as well as strains/genotype involved in causing disease. In this study, a multilocus sequence analysis was carried out to determine the genetic diversity of *A. hydrophila* isolated from fish in Uganda based on five housekeeping genes.

2 | MATERIALS AND METHODS

2.1 | *Aeromonas hydrophila* isolates, biochemical characteristics and GenBank sequences

Nine strains of *A. hydrophila* isolated from Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*) from selected districts of Uganda during a previous study (Wamala et al., 2018) were included in this study (Table 1). Stock cultures of the isolates which were previously stored at −80°C in BHI broth containing 30% glycerol (v/v) were recovered, grown on BHI agar at 30°C for 24 hr, and their biochemical characteristics were determined as previous described (Abbott, Cheung, Kroske-Bystrom, Malekzadeh, & Janda, 1992) (Janda, Abbott, Khashe, Kellogg, & Shimada, 1996) (S. L. Abbott, Cheung, & Janda, 2003). The bacteria were maintained on agar plates in addition to storage at −80°C. Gene sequences of several other *Aeromonas hydrophila* strains and other species of the genus *Aeromonas* and other selected bacteria genera obtained from GenBank were included in the study for comparative genome analysis. Isolates from this study were grouped based on species of fish host, production system of the host and site or district of isolation for the purpose of comparative genome analysis. Details of the bacterial strains and genome sequences included in the analysis are presented in Table 1.

2.2 | Selection of housekeeping genes and primers

Housekeeping genes and the respective PCR primers sets for amplification of each gene were selected according to previous studies (Martino et al., 2011; Yáñez et al., 2003), with a preference to genes involved in DNA/RNA processing function and fulfilling the following criteria: presence as a single copy in all strains, presence of a conserved and variable sequence, wide distribution across the chromosome and are under neutral or negative selection pressure. Accordingly, housekeeping genes including *gyrB*, *gltA*, *metG*, *ppsA* and *recA* were used for MLSA analysis in this study. Nearly full-length of the 16S *rRNA* gene was also included for analysis. Details of selected gene loci and corresponding oligonucleotide primers are shown in Table 2.

TABLE 1 Bacterial strains and sequence accession numbers included in this study

Bacteria strain	Source	Sequence accession no.	Reference
Aeromonas hydrophila 02	*Clarias gariepinus*/Uganda	[a]	This study
A. hydrophila 08	*C. gariepinus*/Uganda	[a]	This study
A. hydrophila 11	*O. niloticus*/Uganda	[a]	This study
A. hydrophila 15	*C. gariepinus*/Uganda	[a]	This study
A. hydrophila 18	*C. gariepinus*/Uganda	[a]	This study
A. hydrophila 19	*C. gariepinus*/Uganda	[a]	This study
A. hydrophila 24	*Oreochromis niloticus*/Uganda	[a]	This study
A. hydrophila 25	*O. niloticus*/Uganda	[a]	This study
A. hydrophila 27	*O. niloticus*/Uganda	[a]	This study
A. hydrophila sub spp. *hydrophila* strain ATCC 7966		CP000462.1	(Seshadri et al., 2006)
A. hydrophila subsp. *Ranae*		NR_042518.1	(Kupfer et al., 2006)
A. hydrophila strain Marseille-P845		LT223616.1	(Raoult, Unpublished)
A. caviae ATCC 15468		NR_029252.1	(Ruimy et al., 1994)
A. salmonicida subsp. *salmonicida* A449	Brown trout/France	CP000644.1	(Reith et al., 2008)
A. salmonicida subsp *achromogenes* strain 6263/4/5		NR_037011.1	(A. J. Martinez-Murcia et al., 1992)
A. schubertii WL1483	Snakehead fish/China	CP013067.1	(Liu et al., 2016)
A. schubertii ATCC 43700	Human/USA	NR_119043.1	(Ruimy et al., 1994)
A. caviae ATCC 15468		NR_029252.1	(Ruimy et al., 1994)
A. sobria ATCC 43979		NR_119044.1	(Ruimy et al., 1994)
A. veronii B565	Pond sediment/China	CP002607.1	(Li et al., 2011)
A. veronii bv. *veronii* ATCC 35624		X60414.2	(A. J. Martinez-Murcia et al., 1992)
A. salmonicida subsp. *salmonicida* strain ATCC 33658		KC244777.1	(Diamanka, Loch, Cipriano, Winters, & Faisal, 2014)
A. jandaei strain ATCC 49568		NR_119040.1	(Ruimy et al., 1994)
A. jandaei strain AH12		KU975020.1	(Peepim, Dong, Senapin, Khunrae, & Rattanarojpong, 2016)
E. coli EHEC Strain ATCC43895		Z83205.1	(Ridell et al., 1994)
E. coli str. K-12 substr. MG1655		CP027060.1	(Beaulaurier et al., 2018)
Pseudomonas aeruginosa strain ATCC 27853		AF094719.1	(Galdzicka, Plassmeyer, Blaine, Pienta, & Gillevet, Unpublished)

[a]accession numbers for sequences from the corresponding isolates fall between MG984617 and MG984625; and MH040697 and MH040739.

2.3 | Genomic DNA extraction, PCR and sequencing

To isolate bacterial genomic DNA, pure colonies were first grown on BHI agar for about 24 hr. Thereafter, the QIAamp™ DNA Mini kit (Qiagen, Germany) was used to extract the DNA following the manufacturer's instructions. The concentration and quality of the extracted DNA were checked using 1% agarose gel and a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.,). The DNA was stored at −20°C until use.

The PCR protocol used for amplification of each gene locus is as previously described (Martino et al., 2011). PCR was carried out using the Qiagen PCR Kit (Qiagen, Germany) in a final volume of 25 μl containing: 2.5 μl of 10× reaction buffer (50 mM KCl, 75 mM Tris–HCl (pH 9.0), 2 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$), 0.5 μl 10 mM deoxyribonucleotide mix, 0.2 μl of Taq DNA polymerase, 1 μl of 10 mM of each forward and reverse primer, 2 μl of DNA template and 16.8 μl of sterile ultrapure water. The amplification programme used in a thermocycler (iCycler Bio-Rad Laboratories, CA, USA) was initial denaturation at 94°C for 1 min, followed by 34 cycles of as follows: denaturation at 94°C for 20 s, annealing at 58°C for 30 s and extension at 72°C for 1.5 min. This was followed by a final extension step at 68°C for 10 min. Amplified products were analysed by electrophoresis using CyberSafe™ prestained 1% agarose gels, ran on PowerPac 300 electrophoretic system (Bio-Rad) at 100 Volts for 60 min. The gels were visualized on Safe Imager™ UV trans-illuminator (Invitrogen). The PCR products were purified using QIAquick Gel extraction kits (Quiagen, Germany) following the

TABLE 2 Selected gene loci and oligonucleotide primers

Locus	Gene product	Primer	Sequence (5′-3′)	Amplicon size (bp)	Annealing temp. (°C)
gyrB	DNA gyrase, beta subunit	gyrB_F	GGGGTCTACTGCTTCACCAA	669	59
		gyrB_R	CTTGTCCGGGTTGTACTCGT		
gltA	Citrate synthase I	gltA-F	TTCCGTCTGCTCTCCAAGAT	626	58
		gltA-R	TTCATGATGATGCCGGAGTA		
metG	Methionyl-tRNA synthetase	metG-F	TGGCAACTGATCCTCGTACA	657	57
		metG-R	TCTTGTTGGCCATCTCTTCC		
ppsA	Phosphoenolpyruvate synthase	ppsA-F	AGTCCAACGAGTACGCCAAC	619	60
		ppsA-R	TCGGCCAGATAGAGCCAGGT		
recA	Recombinase A	recA-F	AGAACAAACAGAAGGCACTGG	640	57
		recA-R	AACTTGAGCGCGTTACCAC		
16S rDNA		16S rRNA-27F	AGAGTTTGATCCTGGCTCAG	1465	56
		16S rRNA-1492R	GGTTACCTTGTTACGACTT		

manufacturer's instructions. The quality and quantity of the products were assessed using 1% agarose gel and nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). PCR products were sequenced commercially at GATC Biotech, Germany, in both directions using the same primers as used for PCR. The sequences generated were deposited in the GenBank database with accession numbers ranging from MG984617 to MG984625; and MH040697 to MH040739.

2.4 | Single gene and Multilocus sequence analysis

Raw trace sequence data for both forward and reverse strands of individual gene loci were manually edited for any errors in base calling, trimmed and forward consensus sequence was generated using BioEdit Sequence Alignment Editor Version 7.0.2 (Hall, 1999) For each isolate, five gene sequences including *metG* (657 bp), *recA* (640 bp), *gyrB* (669 bp), *ppsA* (619 bp) and *gltA* (626 bp) were manually concatenated in a head-to-tail manner, according to their physical order (*gyrB-gltA-metG-ppsA-recA*) in the *A. hydrophila* genome, starting and ending at exactly the same positions.

The consensus sequences generated for each isolate were imported into MEGA 7.0.2 (Kumar, Stecher, & Tamura, 2016) where further analysis was performed. Multiple alignments were carried out using the ClustawW2 algorithm. Diversity indices including number of variable sites, constant sites, parsimony informative sites, singletons, 0-, two- and fourfold degenerate sites were determined for individual and concatenated sequences. Pairwise distance, average evolutionary diversity within and between groups, and net evolutionary diversity between groups were computed using Maximum Composite Likelihood model (K. Tamura, Nei, & Kumar, 2004). Sequence alignments for individual and concatenated gene loci were used to construct phylogenetic trees using neighbour-joining (NJ) (Saitou & Nei, 1987) and maximum likelihood (ML) (K. Tamura & Nei, 1993) methods. Statistical support for the resulting nodes in each phylogenetic tree and for all evolutionary analyses was performed by bootstrapping (BT) method with BT values set at 1000 replicates.

2.5 | Ethical approval

This study was approved by the Uganda National Council for Science and Technology, with reference number "A 509".

3 | RESULTS

3.1 | Phenotypic characteristics and amplification of the selected gene loci

All *A. hydrophila* strains isolated in this study produced mucoid tan colonies on Trypticase soy agar when incubated at 30°C for 1–2 days. The majority of the tests performed yielded uniform results across the isolates. Details of the phenotypic characteristics of the isolates are indicated in Table 3. The selected fragments of the five housekeeping genes considered in this study were successfully amplified and sequenced for all the nine isolates, except for the *ppsA* locus, which was not amplified in two of the isolates.

3.2 | Estimates of evolutionary divergence between sequences

With respect to the housekeeping genes, the *ppsA* gene locus was the least variable with only 6.82% variable sites followed by *gyrB* (6.82%) while *gltA* was more diverse with 11.95% of the sites variable. Overall, the 16S *rRNA* gene was the most conserved with only 2.51% variable sites (Table 4). According to the mean pairwise distance of individual gene loci and concatenated sequences, the genetic diversity among strains was high for the majority of the gene loci. The *gyrB* had the lowest diversity (0.031) while the *gltA* gene showed the highest (0.049) among the housekeeping genes used in the MLSA. The 16S *rRNA* gene loci showed the lowest diversity of all the genes examined (Table 5).

Evolutionary divergence over sequence pairs within groups, between groups and net between-group mean distances was determined using concatenated sequences. Evolutionary diversity within

groups showed varying degree of diversity with respect to the different groups. High diversity was observed within fish species, with isolates from catfish and Nile tilapia having within-group mean genetic distances of 0.031 and 0.032, respectively. With regard to geographical locations, a lower diversity was observed among isolates from Buikwe than those from Wakiso which were more diverse. Isolates from cages were more closely related than those from ponds. Comparison of between-group genetic diversity showed that *A. hydrophila* isolates appear to be genetically diverse with respect to fish host, production systems and site of isolation all with between-group mean distance of 0.033 (SE 0.004). However, the net between-group mean distance was lower with respect to fish host species (0.001 SE 0.001) compared to production systems and site of isolation both with net between-group mean distance of 0.014 (SE 0.002) (Table 6).

TABLE 3 Biochemical characteristics of *Aeromonas hydrophila* isolated in this study

	Isolate number								
Test	**2**	**8**	**11**	**15**	**18**	**19**	**24**	**25**	**27**
Gram stain	–	–	–	–	–	–	–	–	–
Motility	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Citrate utilization	+	–	+	+	+	+	+	–	–
Indole production	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+
Urease	–	–	–	–	–	–	–	–	–
Gelatinase	+	+	+	+	+	+	+	+	+
VP test	+	+	+	+	+	+	+	+	+
Esculin hydrolysis	+	+	+	–	+	+	+	+	+
TSI (growth on tsi)	+	+	+	+	+	+	+	+	+
H_2S production	–	–	–	–	–	–	–	–	–
OPNG	+	+	+	+	+	+	+	+	+
Gas from glucose	+	+	+	+	+	+	+	+	+
Acid from									
Cellobiose	–	–	–	–	–	–	–	–	–
Arabinose	+	+	–	+	+	–	+	+	–
D-Maltose	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+
Hemolysis	B	β	B	β	β	β	B	β	β
Amp resistance	+	+	+	+	+	+	+	+	+

Note. β, β haemolytic; TSI, triple sugar iron; VP, Voges–Proskauer; Amp, Ampicillin; OPNG, ortho-Nitrophenyl-β-galactoside.

In phylogenetic analysis, both neighbour-joining (NJ) and maximum likelihood (ML) methods generally produced similar tree topologies for the different gene loci and the concatenated sequences. However, some variations were observed in branch length and the strength of clustering as seen from differences in bootstrap (BS) values at branch nodes. Tree topologies for individual gene loci (Figure 1a-f) were inconsistent in discriminating isolates according to their sources especially for *ppsA* and *gltA* gene loci. However, increasing consistence was observed with *metG*, *gyrB* and *recA* in clustering isolates according to their sources. These loci clustered all *A. hydrophila* isolates from cages in Buikwe together. However, according to the *ppsA* and *gltA* gene loci, these isolates were clustered with isolates from other sources. The 16S rRNA gene had the poorest resolution in that it nearly clustered all isolates together (Figure 1a).

The concatenated sequence tree (Figure 1g) revealed two distinct clusters according to isolate sources: tilapia cages from Buikwe and catfish ponds from Wakiso. This was the most consistent in resolving isolates according to their sources supported by higher BS values as compared to those of individual gene trees. However, a few isolates belonging to catfish from ponds from Wakiso were placed away from their major cluster. Isolate 11, the only isolate from tilapia from the wild (Lake Victoria), clustered closely to isolate 15 (isolated from catfish in ponds from Wakiso) in all cases except for the *ppsA* gene tree. These two isolates were also clustered together in the concatenated phylogenetic tree separated by *A. veronii* strain B565 from the rest of *A. hydrophila*. In almost all cases, other *Aeromonas* species including *A. veronii*, *A. caviae*, *A. salmonicida* and *A. schubertii* with gene sequences obtained from the GenBank were clustered closer to *A. hydrophila* unlike *Escherichia coli* and *Pseudomonas aeruginosa* strains which were placed furthest as expected for the out-groups.

TABLE 4 Sequence statistics for the six gene loci and the concatenated sequences

Gene Loci	Length	No. of isolates	Parsimony informative sites (Pi)	No. of conserved sites (C)	Number of variable sites (V)	Singleton (S)	0-fold degenerate sites	2-fold degenerate sites	4-fold degenerate sites	Total sites
16S rDNA		9	11	1102	29	18	743	183	195	1154
recA		9	33	574	58	25	403	100	112	635
gyrB		9	39	605	46	7	402	104	121	655
gltA		9	58	545	74	16	409	98	100	619
metG		9	47	574	64	17	394	90	114	643
ppsA		7	32	555	41	9	365	73	115	601
Concatenated sequence		9	210	2865	316	103	2011	456	572	3335

TABLE 5 Pairwise distance ranges and means

Gene Loci	Length	No. of isolates	Pairwise distance range	Mean P-distance (*SE*)
16S rDNA	1154	9	0.000–0.010	0.003 (0.001)
recA	635	9	0.000–0.060	0.032 (0.005)
gyrB	655	9	0.000–0.055	0.031 (0.006)
gltA	619	9	0.000–0.118	0.049 (0.009)
metG	643	9	0.000–0.086	0.042 (0.006)
ppsA	601	7	0.000–0.048	0.033 (0.005)
Concatenated sequence	3335	9	0.001–0.064	0.032 (0.004)

Notes. The number of base substitutions per site between sequences is shown. Analyses were conducted using the maximum composite likelihood model. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.
Mean P-distance, mean pairwise distance.

4 | DISCUSSION

Characterization of disease-causing agents is crucial for proper treatment and control of their associated diseases. Various approaches have therefore been used to delineate a number of bacterial pathogens including the genus *Aeromonas* with varying success. In this study, a multilocus sequence analysis (MLSA) was used to determine the genetic diversity of *A. hydrophila* isolated from fish in Uganda. A total of nine (9) isolates from different fish species (catfish and Nile tilapia), production systems (cages, ponds and wild) and geographical locations (Buikwe, Mukono and Wakiso) were included in this study and characterized using *recA*, *gyrB*, *metG*, *ppsA* and *gltA* gene loci.

The different gene loci used showed varying degrees of discriminatory power in the phylogenetic analysis of *A. hydrophila*. The *recA*, *gyrB* and *metG* gene loci had a better resolution in clustering the isolates according to their sources as compared to the *ppsA* and *gltA* gene loci. Previous studies have shown similar results with the *recA* and *gyrB* (Beaz-Hidalgo et al., 2009; Antonio J. Martinez-Murcia et al., 2011), and the *metG* gene loci, being very useful markers in the phylogenetic analysis of the genus *Aeromonas* and other bacteria. These genes have been shown to have both conserved and highly variable regions among the protein-coding housekeeping genes that make them more suitable for phylogenetic studies. The *gyrB* gene in *Aeromonas* has shown higher substitution rate, 6× that of the 16S *rRNA* gene (A. J. Martinez-Murcia et al., 1992; Yáñez et al., 2003). This is due to the chronometric nature of the *gyrB* gene which although relatively conserved as it encodes a housekeeping protein, it is subject to a degenerative code which allows silent mutations to occur. However, in a different study, the *gyrB* gene could not distinguish between *Vibrio harveyi* and *V. campbellii* (Thompson, Gomez-Gil, Vasconcelos, & Sawabe, 2007) indicating that this gene may not be useful in the phylogenetic analysis of some bacteria. Although the *gltA* and *ppsA* gene loci were less discriminatory in this study with respect to isolate source, these genes have produced more reliable results in delineation of the genus *Aeromonas* according to previous studies (Martino et al., 2011). Indeed even in this study, the *ppsA* and *recA* loci discriminated all *A. hydrophila* strains from other *Aeromonas* species included in this study to a greater extent compared to other gene loci where *gyrB*, *gltA* and *metG* loci were not able to delineate, of course variably, some *A. hydrophila* from *A. veronii*, *A. schuberti* and *A. salmonicida* subsp. *salmonicida*. Phylogenetic analysis using the 16S *rRNA*, which was nearly full length, showed the lowest discriminatory power in resolving isolates of *A. hydrophila* of different sources with nearly all isolates clustered together. This gene has shown similar results in a number of studies (Martínez-Murcia et al., 2005). High differences in sequence diversity of the 16S *rRNA* gene in genus *Aeromonas* have been reported by A. J. Martinez-Murcia et al. (1992). This gene has multiple heterogeneous copies within the genome (Dahllöf et al., 2000; Fogel et al., 1999; Morandi et al., 2005) that could evolve differently in the same organism (Ueda, Seki, Kudo, Yoshida, & Kataoka, 1999) in addition to being highly conserved in genus *Aeromonas* in particular (A. J. Martinez-Murcia et al., 1992). This could partly explain the observed results. Therefore, as indicated by several other authors such as Stackebrandt and Ebers (2006), the 16S *rRNA* gene is not a suitable marker for evaluating the genetic diversity of genus *Aeromonas*. This is especially true in this study, just like several other studies used a nearly full-length sequence of the 16S *rRNA* gene as it is recommended (Rossello-Moraa & Marine, 2015) but still failed to discriminate between the isolates. The gene, however, defined the isolates

TABLE 6 Diversity estimation between groups based on concatenated sequences

Group		Average evolutionary diversity over sequence pairs		
		Within-group mean distances (SEs)	Between-group mean distances	Net between-group mean distances
Fish species	Catfish	0.031 (0.004)	0.033 (0.004)	0.001 (0.001)
	Tilapia	0.032 (0.004)		
Production system	Cages	0.002 (0.001)	0.033 (0.004)	0.014 (0.002)
	ponds	0.037 (0.004)		
District	Buikwe	0.002 (0.001)	0.033 (0.004)	0.014 (0.002)
	Wakiso	0.037 (0.004)		

Note. The number of base substitutions per site from averaging all sequence pairs within and between groups is shown. For net evolutionary divergence between groups, the number of base substitutions per site from estimation of net average between groups of sequences is shown. Standard error estimate (SE is shown in parentheses and was obtained by a bootstrap procedure with 1000 replicates. Analyses were conducted using the maximum composite likelihood model. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.

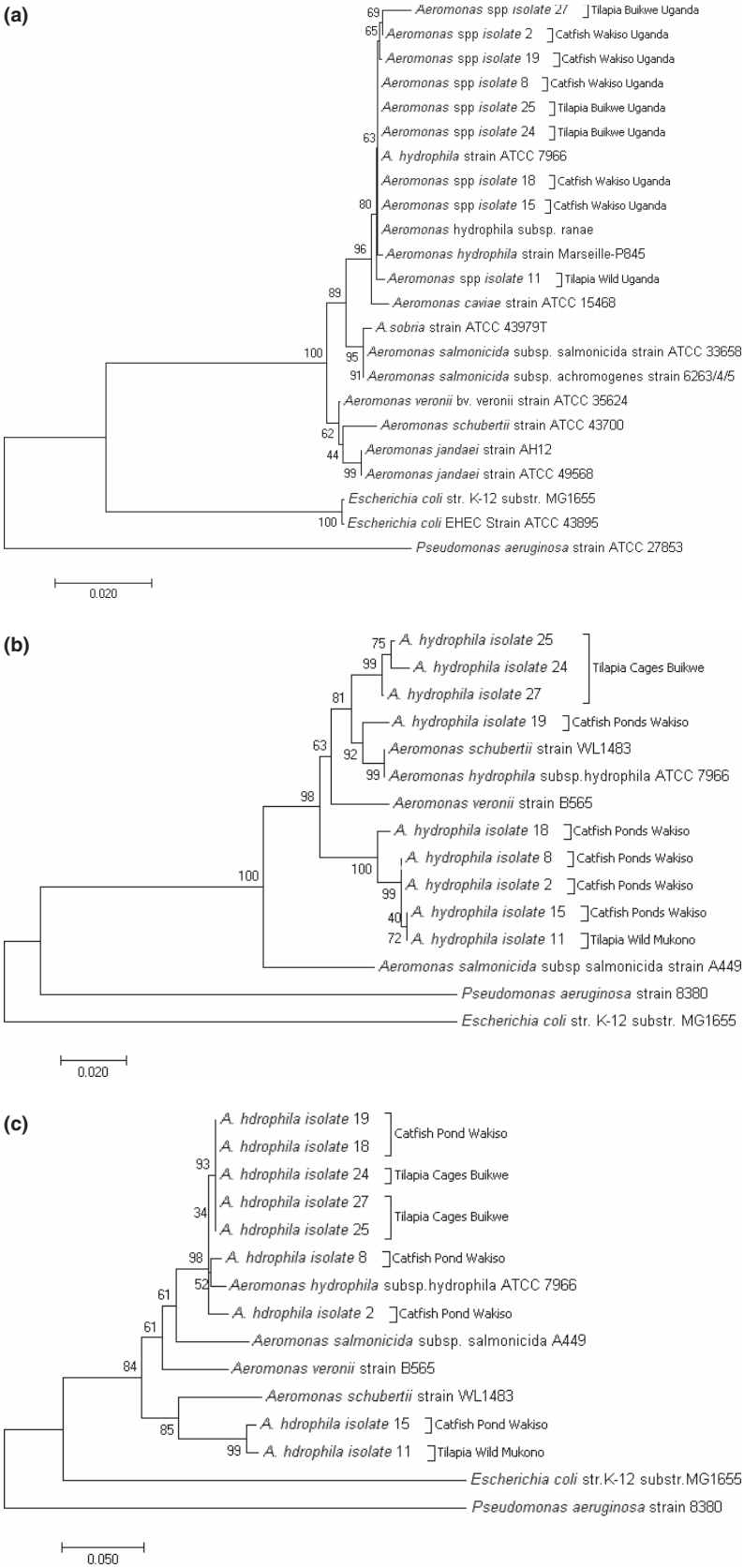

FIGURE 1 a-g, Evolutionary relationships of *Aeromonas hydrophila* from fish in Uganda. The trees are based on individual gene locus and the concatenated sequences as inferred by the neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. All positions containing gaps and missing data were eliminated. a) 16S rRNA gene tree, b) gyrB gene tree, c) gltA gene tree, d) metG gene tree, e)ppsA gene tree, f) recA gene tree and g). Concatenated sequence tree

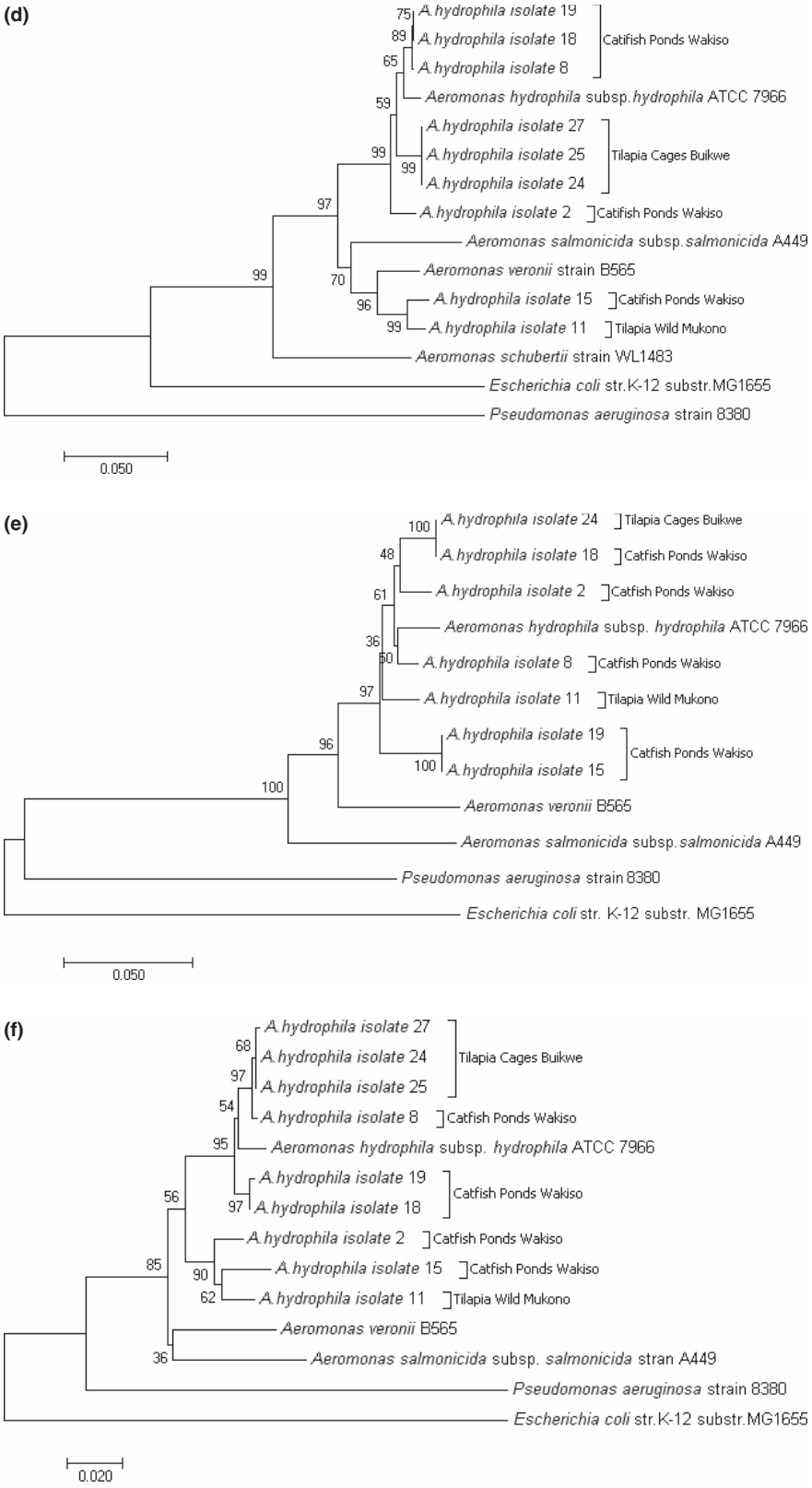

FIGURE 1 Continued.

clearly at genus level as reported by Kupfer et al. (2006) as well as to species level which is consistent with the routine taxonomic application of this gene for bacterial species identification (Figure 1a).

The MLSA had a better discriminatory power in discriminating between *A. hydrophila* isolates by placing more isolates in clusters according to their origin (Figure 1g). This is a similar finding by several

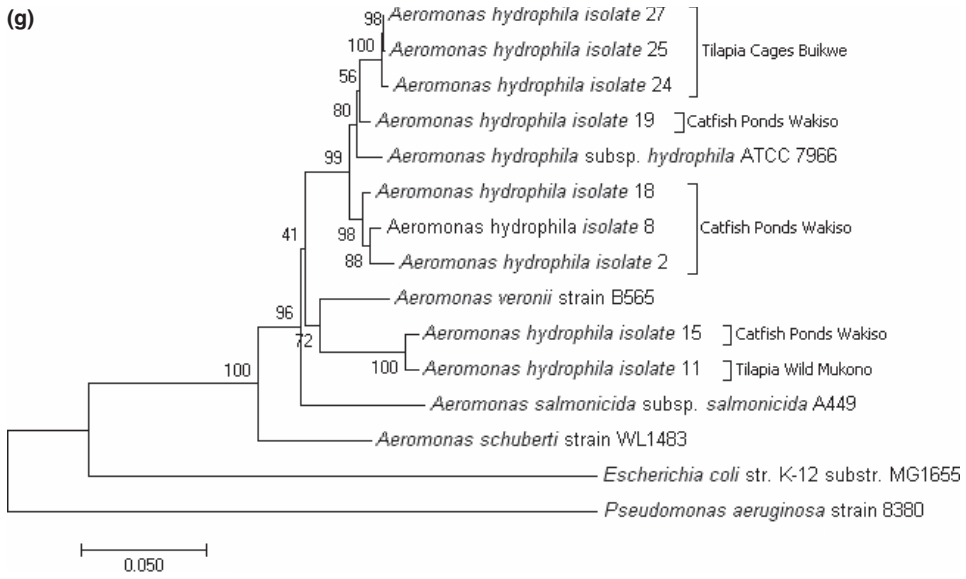

FIGURE 1 Continued

other authors who showed that MLSA is a strong approach for delineating *Aeromonas* isolates (Antonio J. Martinez-Murcia et al., 2011) and other bacteria including *E. tarda* (Abayneh et al., 2012), *Mycobacterium* (Macheras et al., 2011) and *Vibrio* species (Martens et al., 2008). This has been reported to be due to the fact that molecular information is analysed from a wider proportion of the genome in MLSA compared to that from individual gene loci. This results in higher discriminatory power with lower sensitivity to phylogenetic incongruence due to horizontal gene transfers (Vinuesa, 2010). Use of several markers as in MLSA avoids bias from single genes (Glaeser & Kämpfer, 2015) and provides a powerful tool in classifying bacteria (Clarke, Diggle, & Edwards, 2002). However, use of several gene loci may mask the evolutionary history of individual genes (Kämpfer & Glaeser, 2012; Lapierre, Lasek-Nesselquist, & Gogarten, 2014). In our case, the MLSA, supported by the *gyrB*, *recA* and *metG*, shows that the distribution of the isolates into their respective clusters does not result from the allelic diversity of a single gene but more likely the whole genome. Increase in the number of gene loci increases the resolution of the analysis by joining the combined capacities of all molecular clocks. Despite its robustness and higher resolution, the MLSA in the current study failed to discriminate *A. veronii* from *A. hydrophila* as *A. veronii* was within the *A. hydrophila* cluster in three of the individual gene loci making-up the MLSA (*gyrB*, *gltA* and *metG*) suggesting that these gene loci may not be appropriate for *A. veronii* delineation.

The close relationship between *A. hydrophila* and *A. schubertii* in the *gyrB* gene tree could be an indication that some genes evolve in a similar manner in different species of the genus *Aeromonas* and this could be due to similar host and/or environmental adaptations. In a study by Thompson et al. (2007), clustering of different species within genus *Vibrio* with the *gyrB* and *recA* gene loci was also reported. In their study, the *gyrB* gene had no taxonomic resolutions unlike in many other studies. Alternatively, the clustering of other *Aeromonas* species close to *A. hydrophila* could indicate misidentification in previous studies as it has been shown that species under *A. hydropila* complex can easily be misidentified even at genotypic identification. Carnahan and Joseph revealed that there is an increasing number of taxa ascribed to genus *Aeromonas* with over 20 species described; however, in some cases, the validity of the designation is not universally accepted (Carnahan & Joseph, 2005). In the case of *Aeromonas*, 16S *rRNA* gene sequences indicated that the genus is composed of a very tight group of species, some of them differing by only a few nucleotides (A. J. Martinez-Murcia et al., 1992), yet this is the gene commonly used in genotypic bacteria identification.

The reference strains clustered together with some Ugandan strains in several tree topologies from different gene loci and the concatenated trees. This could indicate that the Ugandans strains are genetically very similar to this type strain, as further evidenced by the identification obtained by BLAST searches using the 16S *rRNA* nearly full-length gene in EZBioCloud (Yoon et al., 2017).

The high genetic diversity observed within and between the different groups could indicate that the isolates from the different groups originated from different sources and could represent different outbreaks. The genus *Aeromonas* comprises several well-separated groups of strains, each strain being highly divergent from the others and this could explain the taxonomic differences (Martino et al., 2011). The evolutionary process in the genus *Aeromonas* has resulted in exceptionally high genetic diversity (Roger, Marchandin, Jumas-Bilak, Kodjo, & Lamy, 2012). In ponds, the high genetic diversity observed could indicate that *A. hydrophila* infecting fish on the different farms originated from different sources. A similar scenario could explain the high diversity observed in isolates from Nile tilapia and catfish as they were also from different farms/sites. However, a lower diversity is observed in cage isolates and this could be due to the fact that all isolates were from the same farm indicating that they are clonal complexes arising from a common source of infection. On the other hand, it may be that fish living in ponds may get

exposed to *A. hydrophila* from more different local sources than fish living in net pens.

Although several previous studies indicated that isolates from different sources were clonal suggesting a common origin, our results suggested that the emergence of clonal descents among the analysed *Aeromonas* isolates was limited. This result could, however, be due to the inability of the five gene loci analysed to provide enough information on longer timescales resulting in failure to resolve interrelationships among the lineages corresponding to clonal complexes. Increasing the number of gene loci could give a different picture; however, in a study with *Neisseria meningitis*, increasing the number of analysed loci from 7 to 20 did not resolve the clonal relationships among the strains (Zadoks, Schukken, & Wiedmann, 2005). This may, however, be different for other bacteria.

The deviations observed with some individual gene tree topologies could suggest variations in the evolutionary history of these genes leading to contradicting phylogenetic signals. Phylogenetic incongruence in bacteria may be a result of recombination events (Abayneh et al., 2012; Zadoks et al., 2005), such as horizontal gene transfers (Vinuesa, 2010). *Aeromonas* being a commensal bacterium is likely to undergo rapid recombination events (Zadoks et al., 2005).

5 | CONCLUSIONS AND RECOMMENDATIONS

Aeromonas hydrophila infecting fish in Uganda is of diverse genotypes as evidenced by MLSA. The infections occurring at different farms could represent separate outbreaks arising from different sources of infection. The high genetic diversity within groups indicates that different bacteria from the various sources could be responsible for a single outbreak. The *metG*, *recA* and *gyrB* gene loci had a higher resolution in clustering isolates according to their sources compared to the *ppsA*, *gltA* and the 16S *rRNA* genes. This implies that some genes provide more useful genetic information in phylogenetic analysis of *A. hydrophila* and probably a similar situation for other bacteria. Proper selection of genes and gene loci for such phylogenetic studies is therefore important for the quality of the results. In this study, the MLSA had the highest discriminatory power in clustering *A. hydrophila* isolates according to their sources. We, therefore, further confirm that MLSA is a robust and reliable method for delineation of *A. hydrophila* in epidemiological investigations. Efforts to minimize spread of the bacteria across sources should be emphasized to control infections of mixed genotypes. Serological types of the isolates should be determined especially when isolates are obtained from area where such information is scanty.

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