Off farm commercial culture, amino acid composition and COI and 16S rDNA sequence based identification of fish live food tubificid worms

A thesis submitted to the Department of Fisheries, University of Dhaka
in partial fulfillment of the requirement for the degree of
Master of Science (MS) in Fisheries

Submitted by

Examination roll: 823
Registration number: 2011-112-787

MS session: 2015-2016
Registration session: 2011-2012

Department of Fisheries
University of Dhaka, Dhaka-1000
Bangladesh

February 2017

Acknowledgements

Thanks to ALMIGHTY ALLAH for giving me strength and ability to understand learn and accomplished my thesis work timely.

I express heartfelt gratitude and appreciation to my reverent teacher and research supervisor Dr. Mahmud Hasan, Professor, Department of fisheries, University of Dhaka for his valuable guidance, keen interest, encouragement at various stages of my thesis work and giving me a lot of new ideas about the study and helping to write thesis paper.

Special thanks to my respected co-supervisor Dr. Mohammad Shamsur Rahaman, Associate Professor, Department of Fisheries, University of Dhaka whose guidance, encouragement, suggestion and very constructive criticism has contributed immensely to the evolution of my ideas on the thesis work.

I express my deep sense of gratitude to all of my respected teachers especially Dr. Kaniz Fatema, Chairperson, Professor, Department of Fisheries, University of Dhaka and Chairman of the examination committee Dr. Md. Ghulam Mostafa, Professor, Department of Fisheries, University of Dhaka for their assistance.

I am glad to acknowledge Md. Inja-Mamun Haque for his constant support at various stages of my thesis work.

I thank my family members for encouragement and financial support to complete thesis work.

I wish to thank Ministry of Education, Govt. of the Peoples Republic of Bangladesh for their financial support to conduct this study. We are grateful to Alhajj Firoz Khan, Proprietor, Maa Fatema Fish Hatchery, Jessore, Bangladesh for providing land for the construction of the tubificid worm culture systems and continuous water supply.

I would like to acknowledge Ministry of Science and Technology for giving me the National Science and Technolgy fellowship.

Author

12 February 2017

Abstract

Tubificides (Tubificidae) are one of the best quality live aquaculture foods widely used in the nursing of spawn to fry and rearing of fry to fingerling. Tubificid worms also used as live food in aquarium / ornamental fish. Few studies have been undertaken on the on farm culture of fish life food tubificid worms. However, off farm commercial culture at farmer level has never been studied. Worms are not identified at species level by using molecular techniques particularly COI gene and 16S rDNA sequence found in Bangladesh. This study, therefore, was designed to test the effects of three wet mediums i.e., cattle blood, rice gruel and subsurface clean water on the yield, amino acid composition and COI gene and 16S rDNA sequence based identification of the fish live food tubificid worms found in Bangladesh.

Tubificid worms were brought from the local collectors and cultured commercially in newly constructed culture raceways at Maa Fatema Fish Hatchery, Dalmil Jessore. Tubificid worms were also collected from Dhaka and Mymensingh to identify at species level.

In the present study mixture of 30% Soybean meal, 40% mustard oil cake and 30% mud were used as media ingredients and cattle blood, rice gruel and subsurface clean water were used as the wet mediums. Amino acid profiles of the wild and cultured worms, media ingredient wetted in cattle blood, rice gruel and subsurface water was also determined in this study. Besides culture techniques, molecular techniques has been performed by using COI and 16S rDNA sequencing to identify tubificid worms at species level.

The highest yield of tubificid worms (683.68 ± 3.86 mg cm^{-2}) was harvested from the treatment where media ingredients were wetted in cattle blood while the lowest yield (584. 38 ± 1.41 mg cm^{-2}) was found in the treatment in which the media ingredients were wetted in subsurface clean water (Figure 1). Rice gruel wetted media resulted in the yield of worms (615.63 ± 3.66 mg cm^{-2}) that was significantly different from other two treatments.

Worms raised in the media wetted in blood had nearly 58% protein followed by rice gruel (55%) and water (53%). Similarly highest level of fat (13%) was detected in the worms produced in the media wetted in blood followed by rice gruel (12%) and water (11%).

Among 8 essential amino acids (EAA) detected in the worms, lysine, arginine and leucine were found highest level in the worms raised in the media ingredients wetted in

blood followed by water and rice gruel. Among non-essential amino acids (NEAA), worms raised in the media wetted in blood had the highest level of glycine followed by wild worms. Level of glycine was similar in the worms raised in the media wetted in rice gruel. Alanine and glutamic acid followed similar trends in occurrence.

Three samples of tubificid worms, denoted as TD, TM, TJ collected from Dhaka, Mymensingh and Jessore (cultured), respectively, selected for sequencing by COI and 16S rDNA gene. COI gene identified tubificid worms TD sample as *Tubifex tubifex,* TM as *Tubifextubifex* and TJ as *Branchiura sowerbyi* while 16S rDNA gene identified tubificid worms as *Limnodrilus hoffmeisteri* for all 3 samples.

Multiple sequence alignment was performed to find out the polymorphic sites among the sequenced worms. After comparing the obtained sequence of COI gene it is observed that 154 of 630 sites are polymorphic and dissimilarities among 3 tubificid sample (TD,TM,TJ) 24.22%. From the multiple sequence alignment of 16S rDNA gene sequencing, is was observed that, total 49 of 363 sites were polymorphic and dissimilarities among 3 tubificid sample (TD, TM, TJ) were 13.50%.

Through this study, identification of tubificid worms at species level and off farm commercial culture of tubificid worms has been conducted for the first time in Bangladesh.

Table of contents

Chapter	Title			Page
	Acknowledgements			ii
	Abstract			iii
	Table of Contents			V
	List of Tables			X
	List of Figures			xii
	List of Plate			xv
	List of Flow charts			xvi
	List of Symbols and Abbreviations			xvii
1	Introduction			1-15
	1.1	Background		2
	1.2	Worm Biology		2
		1.2.1	Oligochaete worm	2
		1.2.2	Tubificid worms	3
		1.2.3	Taxonomy of tubificid worms	4
		1.2.4	Feeding	5
		1.2.5	Habitat	5
		1.2.6	Reproduction	6
	1.3	Media ingredients		7
		1.3.1	Soybean meal	7
		1.3.2	Mustard oil cake	7
		1.3.3	Mud	7
	1.4	Molecular identification		8
		1.4.1	COI gene	8
		1.4.2	16S rDNA	10
		1.4.3	PCR	11
		1.4.4	Phylogenetic analysis	13
	1.5	Rational		14
	1.6	Problem statement		14
	1.7	Research needs		14
	1.8	Objectives		15

2	Materials and Methods			17-43
	2.1	Experimental animals and procedures		17
	2.2	Research location		17
	2.3	Experimental system		17
	2.4	Study design		17
	2.5	Media combination		19
	2.6	Collection of media ingredients and wet mediums		19
	2.7	Media supply		19
	2.8	Culture technology		20
	2.9	Inoculation of the worms		20
	2.10	Renewal of the culture system		20
	2.11	Water flow		20
	2.12	Harvest		24
	2.13	Laboratory assay		24
		2.13.1 Proximate composition		24
		2.13.2 Estimation of moisture content		24
		2.13.2.1 Procedure		24
		2.13.2.2 Calculation		24
		2.13.3 Estimation of protein content		26
		2.13.3.1 Principle		26
			2.13.3.1.1 Reagent preparation	26
			2.13.3.1.2 Procedure	26
			2.13.3.1.3 Calculation	27
		2.13.4 Estimation of fat content		28
		2.13.4.1 Principle		28

	2.13.4.2	Procedure	28
	2.13.4.3	Calculation	28
2.13.5	Estimation of ash content		29
	2.13.5.1	Principle	29
	2.13.5.2	Procedure	29
	2.13.5.2	Calculation	29
2.13.6	Estimation of TDF (total dietary fiber)		29
	2.13.6.1	Principle	29
	2.13.6.2	Reagent	29
	2.13.6.3	Procedure	30
	2.13.6.4	Calculation	30
2.13.7	Calculation of Carbohydrate		30
2.13.8	Amino acid profiling		31
2.13.9	Chemical Composition of Mud		32

2.14 Molecular identification 35

2.14.1	Automated DNA Extraction		35
2.14.2	Measurement of the Concentration of Extracted DNA		35
2.14.3	Conventional PCR Amplpification		35
2.14.4	Agarose Gel Electrophoresis		37
2.14.5	PCR purification		
	2.14.5.1	Purification of Amplicon	39
2.14.6	Measurement of the Concentration of the Amplicon		39
2.4.7	Sequencing Based Identification		39
	2.14.7.1	Sequencing Reaction	39
	2.14.7.2	Sequencing Alignment and Bioinformatics analysis	40
	2.14.7.3	Bioinformatics tools	40

	2.14.8	Phylogenetic Analysis	42
		2.14.8.1 Construction of phylogenetic tree	42
	2.15	Data analysis	43
3		Results	45-64
	3.1	Yield of tubificid worms	45
	3.2	Proximate composition of the worms	45
	3.3	Amino acid profiles of the wild and cultured worms	46
	3.4	Chemical composition of mud	47
	3.5	Molecular identification of Tubificid worms	48
	3.5.1	COI sequence based identification	48
	3.5.1.1	PCR amplification	48
	3.5.1.2	Sequence output	48
	3.5.1.3	DNA sequence alignment	50
	3.5.1.3.1	Alignment among sample TD_Tubifex_COI, TM_Tubifex_COI and TJ_Tubifex_COI	50
	3.5.1.3.2	Alignment between TM_Tubifex_COI and TJ_Tubifex_COI	51
	3.5.1.3.3	Alignment between TD_Tubifex_COI and *Tubifex tubifex*	52
	3.5.1.3.4	Alignment between TM_Tubifex_species and *Tubifextubifex*	53
	3.5.1.3.5	Alignment between TJ_Tubifex_species and *Branchiura sowerbyi*	54
	3.5.1.4	Phylogenetic analysis	55
	3.5.2	16S rDNA sequence based identification	56
	3.5.2.1	PCR amplification	57
	3.5.2.2	Sequence output	57

	3.5.2.3	DNA sequence alignment	59
		3.5.2.3.1 Alignment among Sample TD_Tubifex_16SrDNA, TM_Tubifex_16SrDNA and TJ_Tubifex_16SrDNA	59
		3.5.2.3.2 Alignment between TD_Tubifex_16SrDNA and TM_Tubifex_16SrDNA	60
		3.5.2.3.3 Alignment between TD_Tubifex_16SrDNA and *Limnodrilus hoffmeisteri*	61
		3.5.2.3.4 Alignment between TM_Tubifex_16SrDNA and *Limnodrilus hoffmeisteri*	62
		3.5.2.3.5 Alignment between TJ_Tubifex_16SrDNA and *Limnodrilus hoffmeisteri*	63
	3.5.2.4	Phylogentic analysis	64
4	Discussion		66
5	Conclusions and Recommendations		73
	5.1 Conclusions		74
	5.2 Recommendations		74
	References		76
	Appendices		82

List of Tables

Table	Title	Page
1	Combinations of media ingredients in three culture treatments	19
2	Nutrient composition of the media ingredients and wetted media in blood, rice gruel and water	31
3	Nutrient composition of the media ingredients and wetted media in blood, rice gruel and water	31
4	Amino acid profiles of the media ingredients wetted in blood, rice gruel and water sampled after 7 days wetting	32
5	Composition of GoTaq® Hot Start Colorless Master Mix	35
6	Primer sequences used for conventional PCR amplification	37
7	Preparation for conventional PCR	38
8	Temperature and time profile of the thermocycler	38
9	Proximate composition of worms	46
10	Amino acid profiles of the wild and cultured worms in which media ingredients were wetted in blood, rice gruel and subsurface clean water	47
11	Measurement of DNA concentration of 3 samples	47
12	COI sequence based identification of representative tubificid worms from three different region	48
13	Polymorphic sites observed between closely related species TM_Tubifex_COI and TJ_Tubifex_COI	51
14	Polymorphic sites observed between TD_Tubifex_COI and *Tubifex tubifex*	52
15	Polymorphic sites observed between TD_Tubifex_COI and *Tubifex tubifex*	53
16	Polymorphic sites observed between TJ_Tubifex_COI and *Branchiura sowerbyi*	54
17	List of all downloaded species from NCBI GenBank to support the phylogenetic analysis	55
18	16S rDNA sequence based identification of representative tubificid worms from three different region	57

19	Polymorphic sites observed between closely related species TD_Tubifex_16SrDNA and TM_Tubifex_16SrDNA	60
20	Polymorphic sites observed between TD_Tubifex_16SrDNA and *Limnodrilus hoffmeisteri*	61
21	Polymorphic sites observed between TM_Tubifex_16SrDNA and *Limnodrilus hoffmeisteri*	62
22	Polymorphic sites observed between TJ_Tubifex_16SrDNA and *Limnodrilus hoffmeisteri*	63
23	List of all downloaded species from NCBI GenBank to support the phylogenetic analysis	64
24	Inoculum size, cost of production, harvest over 40 day culture duration in comparison to present one.	69

List of Figures

Figure	Caption	Page
1	Tubificid worms	4
2	PCR to amplify the entire mitochondrial genome sequence from small amounts of total genomic DNA (gDNA) isolated from individual nematodes for subsequent direct sequencing.	9
3	16S rDNA gene sequencing method.	11
4	Sequencing of PCR product of Tubificid worms	40
5	Chromatogram of the forward (F) and reverse (R) sequence of isolate TD_Tubifex_COI in FinchTV	41
6	Yield of tubificid worms (mg cm^{-2}) harvested from the media wetted by cattle blood, rice gruel and subsurface water after 40 day culture duration in cemented culture raceway of 0.567 m^2 at farmer level. Bars (mean ± 1 SEM) with different letters indicate significant differences (ANOVA, HSD; $p<0.05$).	45
7	PCR amplification product profiles of COI primer generated from 3 different Samples: TD,TM,TJ. Amplicon size was 710 bp. Here, 1 kb marker was used. Sample ID TD, TD_Tubifex_COI; TM, TM_Tubifex_COI; TJ, TJ_Tubifex_COI. Legend: (-ve) denotes negative control.	49
8	Multiple sequence alignment of COI gene fragment of three Tubifex species TD_Tubifex_COI, TM_Tubifex_COI, TJ_Tubifex_COI where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 160)	50
9	Pairwise sequence alignment of COI gene fragment of closely related species TM_Tubifex_COI and TJ_Tubifex_COI where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 160)	51
10	Pairwise sequence alignment of COI gene fragment of sample TD_Tubifex_COI and GenBank reference *Tubifex tubifex* where black among the red color indicates polymorphic positions.	52

	(CLUSTALW, alignment width 140)	
11	Pairwise sequence alignment of COI gene fragment of sample TM_Tubifex_COI and GenBank reference *Tubifex tubifex* where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 140)	53
12	Pairwise sequence alignment of COI gene fragment of sample TJ_Tubifex_COI and GenBank reference *Branchiura sowerbyi* where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 140)	54
13	The neighbor-joining Phylogenetic tree were based on partial COI gene sequences. The evolutionary distances were compared by Maximum Composite Likelihood method. Number in tree are bootstrap values.	56
14	PCR amplification product profiles of 16S rDNA primer generated from 3 different Samples: TD,TM,TJ Here, 1 kb marker was used. Sample ID TD, TD_Tubifex_16SrDNA; TM, TM_Tubifex_16S rDNA; TJ, TJ_Tubifex_16SrDNA	58
15	Multiple sequence alignment of 16S rDNA gene fragment of three samples TD_Tubifex_16SrDNA, TM_Tubifex_16SrDNA, TJ_Tubifex_16SrDNA where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 100)	59
16	Pairwise sequence alignment of 16S rDNA gene fragment of closely related species TD_Tubifex_16SrDNA and TM_Tubifex_16SrDNA where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 100)	60
17	Pairwise sequence alignment of 16SrDNA gene fragment of TD_Tubifex_16SrDNA and GenBank reference *Limnodrilus hoffmeisteri* where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 80)	61
18	Pairwise sequence alignment of 16SrDNA gene fragment of TM_Tubifex_16SrDNA and GenBank reference *Limnodrilus hoffmeisteri* where black among the red color indicates	62

	polymorphic positions. (CLUSTALW, alignment width 100)	
19	Pairwise sequence alignment of 16SrDNA gene fragment of TJ_Tubifex_16SrDNA and GenBank reference *Limnodrilus hoffmeisteri* where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 100)	63
20	The neighbor-joining Phylogenetic tree was based on partial 16S rDNA gene sequences. The evolutionary distances were compared by Maximum Composite Likelihood method. Numbers in tree are bootstrap values.	65

List of plates

Plates	Caption	Page
1	Media wetting and culture system	21
2	Media placement	22
3	Worms' inoculation	23
4	Worms harvesting	25
5	Determination of protein	33
6	Amino acid profiling	34

List of flow chart

Flow chart	Title	Page
1	Entire procedure of the culture of tubificid worms	18
2	Molecular identification of tubificid worms	36

List of symbols and Abbreviation

Symbols	Details
ANOVA	Analysis of varience
BLAST	Basic Local Alignment Search Tool
CD	Cow dung
COI	Cytochrome c oxidase subunit I
Cyt b	Cytochrome b gene
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
HSD	Honestly Significant Difference
MEGA	Molecular Evalutionary Genetics Analysis
MOC	Mustard oil cake
NCBI	National Centre for Biotechnology information
NFN	Nitrogen free extract
NPN	Non-protein nitrogen
PCR	Polymerase chain reaction
rDNA	Ribosomal Deoxyribonucleic acid
SD	Standard Deviation
SPSS	Statistical Package for the Social Sciences
TAE	Tris-acetate EDTA
TDF	Total dietary fiber
WB	Wheat bran

Chapter 1
Introduction

1.1 Background

Catfishes contribute nearly 12.79% of the total country's fish yield in Bangladesh. Striped catfish *Pangasianodonhypophthalmus* (Sauvage, 1878) is the top 1 culture species in Bangladesh contributing nearly 11.04% of the total fish production (FRSS, 2016). Farming high value catfishes for example, stinging catfish *Heteropneustesfossilis*(Bloch, 1794), walking catfish *Clariasbatrachus*(Linnaeus, 1758) and pabda *Ompok* spp is increasingly becoming popular because of their high market demand. Farming of these catfishes requires live food in the nursing of spawn to fry and rearing of fry to fingerling. Catfish spawn are fed blended tubificid worms while fries are given the whole organism. Sustainable production of catfishes requires reliable supply of good quality hygiene fish seed which in turn depends upon the reliable supply of hygiene produced fish live food tubificid worms that is currently not farmed in Bangladesh. Wild harvest worms cannot meet the growing demand of tubificid worms.

Everyday thousands of cattle are being slaughtered in every city and town. However, the outcome of slaughtering animals' blood is not harvested rather washed out. If harvested this large quantity of blood might have good use in the production of fish live food tubificid worms.

Rice gruel is produced in every household, restaurant and student dormitory. Cattle are fed rice gruel produced in the village household. However, rice gruel produced in the restaurant and student dormitories is drained out. Use of rice gruel as wet medium of the media ingredients enhanced production has been demonstrated in raising tubificid worms by Mariom and Mollah (2012) and Hossain et al. (2012).

Soybean meal (SBM) and mustard oil cake (MOC) have been widely used in fish feed.

SBM contain 40-50% crude protein but lacks cystidine, methionine, calcium, phosphorus and vitamin $B_{complex}$(Hasan, 2003). MOC may contain 28-38 % crude protein and all commonly found amino acids (Sarker et al., 2015).

1.2 Worm biology

1.2.1 Oligochaete worm

The oligochaete worms belong to the class Clitellata (phylum Annelida). Aquatic oligochaetes have been previously considered as freshwater animals, few marine representatives (for example, *Paranais, Tubificoides*, some *Lumbricillus* and *Marionina*) as exceptions. Nowadays, the number of marine tubificids has surpassed the number of freshwater; some of its subfamilies are mainly marine. They are very small, usually not much more than 2 cm in length with a diameter of only a fraction of a millimeter.

Typically, each segment of the body has four bristle bundles (chitin bristles protruding from the body). The setae vary considerably in size and shape, and between families, so they are used to a large extent in identification. The position of one gonad with respect to the next, and the fragments in which they occur are utilized to characterize the families. In the Tubificidae, the type of the male duct is used to characterize genera. Oligochaete are hermaphroditic, with a spacious coelom and a clitellum. The clitellum is a trademark regenerative organ that shows up during sexual development. There are usually a couple sets of male and female gonads, arranged in the front part of the body, the testes are foremost to the ovaries, and the genital products (gametes together with accompanying material) are released through special ducts.

1.2.2 Tubificid worms

Tubificid worms **(Figure 1)**are aquatic invertebrates under the class Oligochaeta and family Tubificidae, commonly known as sludge or sewage worms. Tubificid worm is one of the cosmopolitan freshwater oligochaetes which plays a vital role as supplementary food (live or freeze dried) in intensive aquaculture. Most tubificids have erythrocruorin, a red blood pigment, which can effectively extract oxygen dissolved in the water. They are segmented, bilaterally symmetrical worms with tapering ends (Brinkhurst and Kennedy 1965). Some freshwater forms burrow in the bottom mud and silt; others live among submerged vegetation. The worms lie with heads down and rear ends projecting from tubes and waving vigorously in order to increase aeration. There are two types of Tubificid worms (Phylum Annelida, Order Oligochaeta, Family Tubificidae) which are raised and sold as fish food, the red (tubifex) which has been in the hobby for around 100 years, and the dark Tubifex which is a comparable animal groups that is darker in shading. The amino acid profile of the proteins in Tubifex is pretty good, but the fatty acid profile is quite bad. Therefore, Tubifex fed fishes grow rapidly but do not show as much color as deformities do. Black tubifex is more resistant, more resistant to drying and less prone to cause disease in fish. Tubifex were routinely used as food for fish that rejected food in flakes and when people wanted to condition their fish, that is, to fatten them for breeding.

Figure 1 Tubificid worms

Tubificid worms have been proved as one of the most important live foods for rearing the larvae of hatchery produced catfishes, prawn and ornamental fishes because of having high food value (5575 cal g on the basis of dry weight) that makes them nutritious for fish. Mollah and Ahamed (1989) determined the proximate composition of tubificid worms and found 63.32% crude protein, 28.84% crude lipids and 7.95% ash. Jhingran additionally recommended tubificid worms as a good source of protein and amino acid profile for fish growth. Larvae and fry showed good response in terms of survival and growth rate due to the use of tubificid worms as their food compared to others.

1.2.3 Taxonomy of Tubificid worms

Kingdom: Animalia
- Subkingdom: Bilateria
  - Infrakingdom: Protostomia
    - Superphylum: Lophozoa
      - Phylum: Annelida
        - Subphylum: Clitellata
          - Class: Oligochaeta
            - Subclass: Oligochaeta
              - Order: Tubificida Brinkhurst, 1982
                - Family: Naidae Ehrenberg, 1828
                  - Subfamily: Tubificinae Vejdovsky, 1876

1.2.4 Feeding

Oligochaetes (especially tubificides) are vital as primary consumers, primary decomposers, substrate modifiers, and as sustenance for predators. They may constitute the largest proportion of macrofauna present in natural freshwater systems and are particularly useful as biological indicators of pollution due to their high tolerance to a wide range of variations in environmental quality and also have a life cycle. Therefore, they are only exposed to contaminants from aquatic sources, providing good site specificity. Some facultative vegetation-inhabitants among the Lumbriculidae (e. g., *Lumbriculus*) are likewise ready to swim. The aquatic Tubificidae ingest sediment particles, digesting a species-specific selection ofbacteria, thus mixing the uppermost sediment layer and acceleratingthe oxydation of organic matter (Fisher et al. 1981).Two marine genera (*Olavius* and *Inanidrilus*) feast upon symbiontic microscopic organisms in their body divider while the digestive system is reduced.

1.2.5 Habitat

Aquatic oligochaete species cover a wide range of dissemination and resistance in the aquatic environment compared and other taxa whose distribution is highly restricted. Tubificid worms occur in a wide range of habitats and tolerate a spectrum of environmental conditions (Kaster, 1980; Brinkhurst and Kennedy 1965). Oligochaete worms have colonized an extensive variety of aquatic and semi-aquatic and freshwater, saline and marine benthic groups normally incorporate a few trademark oligochaete animal varieties. These possess a scope of microhabitats in silt, and in addition in aquatic vegetation and dicomposed organic matter. Most species are sedentary Feeding on bacteria, algae and mineral particles rich in organic matter. A few freshwater oligochaete animal groups additionally happen in the meiobenthic fauna of the profundal zone of lakes and can be utilized as markers of contamination. They are the only worms present in the deepest regions of lakes (Mackie, 2001) the depth of a metre or so is the usual habitat for the great majority species belonging to the Naididae (Barnes, 1966).*Tubifex* and *Limnodrilus hoffmeisteri* were the most rich oligochaetes in severly contaminated destinations. Small reddish annelids (3-4 cm long) are found mainly in old canals that have organic detritus, drain with flowing water etc. Aquatic growth environment where rich in organic detritus. They take sediments to obtain nutrition by selectively digesting bacteria in it and absorb nutrient molecules by using their body walls (Rodriguez et. al., 2001) *Tubifex tubifex* (Tubificidae) is particularly interesting because it is an indicator

ofpollution. Therefore, the reduction of organic matter decreases the density of *Tubifex tubifex*.

1.2.6 Reproduction

Oligochaetes are monoecious that implies they have both male and female regenerative framework and oligochaetes are protandrous, as testis develop sooner than ovary. Having a prominent wide band called the clitellum. The two sets of fundamental recepticalson sections 9 and 10. The match of female genital pores on portion 14, and the male genital pores on section 15. *Lumbricus* individuals mate in warm, moist conditions at night. Individuals are found anteriorly and are aligned ventrally. They position the male genital pores near the other clitellum. Clitellum of the Oligochaete secretes the mucosa during the mating process. This mucosa facilitates the transfer of sperm between individuals. Sperm are produced in the testicles, mature in the seminal vesicles, enter the spermatic duct through sperm funnels and exit the male genital pores. The spermatozoa are transferred to the seminal receptacles by external semiannual furrows assisted by the mucosa. This happens for both individuals. The partner's sperm is received and stored by the opposing individual. The companion sperm is received and stored by the seminal receptacles of opposing individuals. Once the sperm is transferred, the worms part ways. In addition to the mucosa, clitellum secretes a band of chitin. It is the initial form of what will be a coccon. The coccon moves forward and external fertilization takes place as the eggs are collected from the female genital pores, as well as the partner's sperm (stored in the seminal vessels). When the coccon is spilled on the anterior end (head) of the animal, it dries and forms the coccon of the final state. The worm is then free to move on. After a few weeks to a couple of months the young worms will leave the coccon. Three tubificid clades (*Naididae, Pristinidae and Opistocystidae*) have involved the surface of sediments and aquatic plants. They are moderately small; a hefty portion of them can swim and even have pigmented eyespots (e.g., *Stylaria* and *Nais*); some others construct temporary slime tubes attached to the substrate (e.g., *Dero* and *Ripistes*). They reproduce mainly by paratomy (forming chains of zooids) alternating it periodically with sexual reproduction. Paratomy also prevails in the "oligoethoid" Aeolosoma, convergingly similar to the naidids. Among other oligoqueous families, asexual reproduction is rare, being represented only by simple fragmentation, or architomy (e.g., *Lumbriculus, Cognettia*, *Bothrioneurum, Aulodrilus*).

1.3Media ingredient

1.3.1Soybean meal

Soybean meal is the byproduct of the extraction of soybean oil. There are several processes, resulting in different products. Soy flour is generally classified for marketing by its raw protein content. There are two main categories of soy flour, "high protein" soy flour with 47-49% protein and 3% crude fiber obtained from dehulled seeds, and "conventional" soy flour with 43-44% protein, which in solvent-extracted soy foods, the oil content is typically less than 2%, while it exceeds 3% in mechanically extracted foods (Cromwell, 2012).It has been considered for a long time as an excellent source of supplementary protein in diets for livestock and poultry. In fact, soy flour is sometimes referred to as the "gold standard" because other sources of protein are often compared to it. Soy meal is rich in high protein edibility, and the protein is made out of a predominant mix of amino acids, building block of body protein for animals and poultry.

Soybean meal is important source of protein used to feed farm animals. It accounts for two-thirds of total world protein production, including all other meals and fishmeal. Its nutritional value is unsurpassed by any other source of vegetable protein and is the standard at which other sources of protein are compared (Cromwell, 1999). Expansion of aquaculture and bans on the use of by-products from slaughterhouses has also been fueled demand for this high quality protein source (Steinfeld et al., 2006).

1.3.2 Mustard oil cake

Mustard oil cakes are by product obtained after the extraction of oil from the seeds. Oil cakes are of two types, edible and inedible. Edible oil cakes have a high nutritional value, especially they have a protein content ranging from 15% to 50% (www Seaofindia.com). Its composition varies depending on its variety, growth condition and extraction methods. Due to their protein content, they are used as animal feed.

1.3.3 Mud

The worms showed good survival, growth and reproduction rates in activated sludge, but only when mixed with sand or river sludge. It seems likely that the addition of sand or sludge to the sludge will decrease the oxygen demand per unit volume of substrate which avoids unfavorable anaerobic conditions and produces a firmer substrate which is more conducive to high rates of growth and reproduction. Laboratory studies (in japan) on the

preference of the substrate of oligochaetes showed that *Branchiura sowerbyi* preferred organic mud than sand, whereas non-susceptible oligochaetes (*Limnodrillus sp*.) had no substrate tropism. In addition, morbidity and mortality of *B. sowerbyi* were observed in breeding in sand. It was also postulated that non-susceptible oligochaetes could act as a biofilter of *T. hovorkai*. Consequently, by replacing the lower substrate of the sludge with sand, it was suggested that a change in the oligochaetic communities of *B. sowerbyi* to non-susceptible oligochaeans was feasible.

1.4 Molecular identification

Recently DNA-based (genotypic) approaches have increasingly been applied to microbial identification and classification. Over the last thirty years, since the discovery of restriction endonucleases, rapid DNA sequencing and polymerase chain reaction (PCR), the analysis of DNA sequence or DNA polymorphisms has become the standard for determining relationships among the bacteria. These methods are quick, accurate, and do not require many cells.

1.4.1 COI gene

Cytochrome c oxidase I (COX1) also known as mitochondrially encoded cytochrome c oxidase I (MT-CO1) is a human-coded MT-CO1 gene. In other eukaryotes, the gene is called COX1, CO1 or COI. The DNA size of the cytochrome c oxidase subunit 1 is about 1548 bp, which has a total T content of 70.2% (Shaikevich and Zakharov., 1993). The cytochrome c oxidase protein (EC 1.9.3.1) is the last enzymatic complex in the respiratory electron transport chain found in the mitochondrial membrane (Valnot et al., 2000). This enzyme acts as a dioxygen activator in aerobic life by transferring the cytochrome c electron reduced to oxygen (Valnot et al. 2000). There are three different subunits of cytochrome c oxidase, COI, COII and COIII. Among the three mitochondrial genes encoding cytochrome c oxidase subunits, COI is the largest and the most conserved among them (Beard et al. 1993).

The mitochondrial cytochrome oxidase 1 (COI) subunit gene is one of the most popular markers for genetic and phylogenetic studies of populations throughout the animal kingdom. The whole procedure of COI gene based identification is given in **Figure 2**. Nevertheless, COI based DNA barcoding sometimes faces problems, in some taxa, such as Porifera, Anthozoa, fungi, Plants. DNA barcoding using the mitochondrial cytochrome *c* oxidase subunit 1 is a reliable method for species identifying (Dawnay et

al., 2006). This gene is much conserved in all species and its rate of evolution is too slow. The two most commonly used genetic loci in species identification are the cytochrome *c* oxidase I gene (COI) and the cytochrome *b* gene (cyt b) (Tobe, 2010).

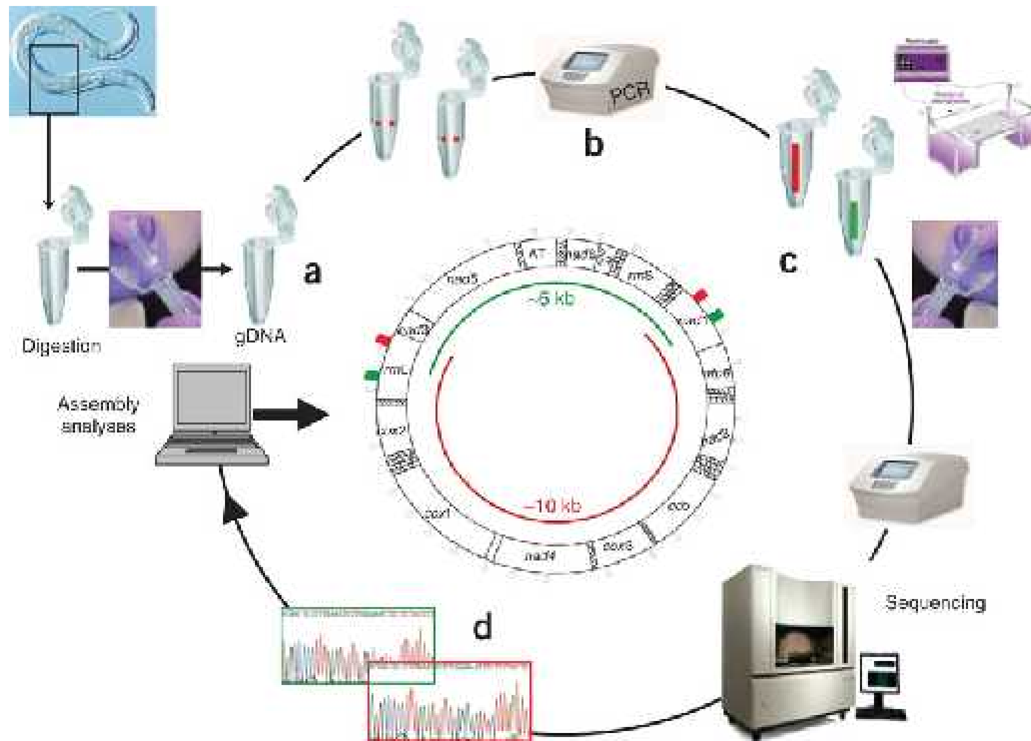


Figure 2 PCR to amplify the entire mitochondrial genome sequence from small amounts of total genomic DNA (gDNA) isolated from individual nematodes for subsequent direct sequencing.

(Source: http://www.nature.com/nprot/journal/v2/n10/full/nprot.2007.358.html)

(**a**) Total gDNA is extracted from individual nematodes using a standard SDS/proteinase K treatment, followed by purification over a mini-column. The internal transcribed spacer regions of nuclear ribosomal DNA are PCR-amplified and sequenced to verify the specific identity of the nematode. (**b**) The entire mitochondrial genome is amplified in two fragments (each ~5–10 kb in size) by long PCR from ~20 ng (~10%) of the total gDNA, employing oligonucleotide primers (red and green pairs; center) constructed to regions in the genes (e.g., *nad*1 or *rrn*L) known to be relatively conserved among selected nematodes. (**c**) Each PCR yields a single amplicon, detected in a 1% agarose gel after ethidium bromide staining. Each amplicon is purified over a mini-spin column, and then sequenced using a 'primer walking' strategy. (**d**) Sequences obtained are assembled and subjected to bioinformatic analyses.

The Cytochrome C oxidase, a mitochondrial protein, located in the inner mitochondrial membrane, and is a key enzyme in the electron transport chain. It therefore plays a central role in aerobic metabolism of eukaryotic organisms. It consist of several units, and cytochrome c oxidase subunit 1 mitochondrial genome is encoded in the catalyst. COI genes, metabolism and its presence in almost all eukaryotes was chosen because of the central role in metabolism.

The cytochrome c-oxidase subunit 1 (COI) mitochondrial gene from the ciliates was first successfully sequenced in species of the genera *Tetrahymena* and *Paramecium* (Class Oligohymenophorea). The COI gene sequence is extremely divergent from other eukaryotes and includes an insert, which is more than 300 nucleotides in length. Hebert and colleagues (Hebert et al., 2003a, 2003b) introduced the use of DNA barcodes as a molecular tool to aid in the discrimination of species using the cytochrome c oxidase (COI) subunit 1 gene.

1.4.2 16S rDNA

The rRNA gene is the most conserved (least variable) DNA in all cells. Portions of the rDNA sequence from distant organisms are remarkably similar. This means that the sequences of distantly related organisms can be aligned accurately, making the real differences easy to measure. For this reason, genes encoding rRNA (rDNA) have been widely used to determine taxonomy, phylogeny (evolutionary relationships), and to estimate species divergence rates among different animals. Therefore, the comparison of 16s rDNA sequence may show evolutionary relationship between microorganisms. A comparison of the genomic sequences of bacterial species showed that the RNA 16S ribosomal RNA (rRNA) gene is highly conserved within a species and between species of the same genus and thus can be used as the new gold standard for the Bacterial specification (Woo et al. 2000).

The use of 16S rRNA gene sequences is one of the most accepted schemes for establishing bacterial identity. The reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes. The moderated way of rRNA arrangements has additionally prompted to the advancement of bland DNA tests for microorganisms. The increased ribosomal quality (rDNA) is subjected to limitation endonuclease absorption; This has

been called ARDRA (Amplified Ribosomal DNA Restriction Analysis). The subsequent limitation part example is then utilized as a unique finger impression for the recognizable proof of bacterial genomes. This method is based on the principle that the restriction sites on the RNA operon are conserved according to phylogenetic pattern (Woese, 1987; Stackebrandt and Goebel, 1994). Phenotypic identification of fermentative microflora is time consuming and often problematic due to ambiguous and overlapping biochemical or physiological traits. The whole procedure of 16S rDNA gene based identification is given in **Figure 3.**

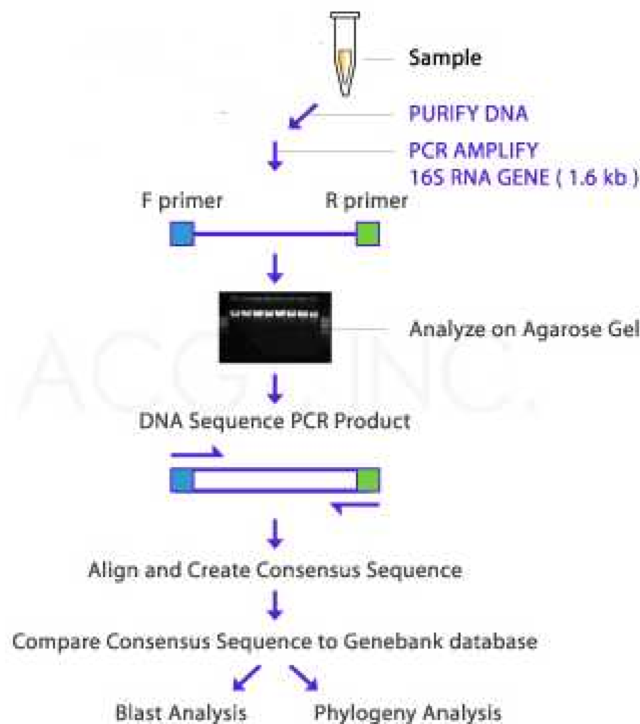


Figure 3 16S rDNA gene sequencing method.

(Source: http://microbiollogy.blogspot.com/2014/08/diagnostic-medical-microbiology_5.html)

1.4.3 PCR

Polymerase chain reaction (PCR) is an efficient and cost-effective way to copy or “amplify” small segments of DNA or RNA. Using PCR, millions of copies of a section of DNA are made in just a few hours, yielding enough DNA required for analysis. This innovative yet simple method allows clinicians to diagnose and monitor diseases using a minimal amount of sample, such as blood or tissue.

Though PCR occurs in vitro, or outside of the body in a laboratory, it is based on the natural process of DNA replication. In its simplest form, the reaction occurs when a DNA sample and a DNA polymerase, nucleotides, primers and other reagents (man-made chemical compounds) are added to a sample tube. The reagents facilitate the reaction needed to copy the DNA code.

In addition to detecting diseases in a sample, PCR enables the monitoring of the amount of a virus present or viral load, in a person's body. In diseases such as hepatitis C or human immunodeficiency virus (HIV) infections, viral load is a good indication of how sick a person may be or how well a person's medicine and treatment is working. Armed with this information, physicians may determine when to start treatment and the person's response to treatment, making treatment personalized to each individual.

There are three clear steps in each PCR cycle, and each cycle approximately doubles the amount of target DNA. This is an exponential reaction so more than one billion copies of the original or "target" DNA are generated in 30 to 40 PCR cycles.

Sample Preparation

Before initiating PCR, DNA must be isolated from a sample. DNA extraction is a multi-step process that may be done manually or with an instrument like the COBAS® AmpliPrep Instrument, the first instrument that prepared samples automatically without human intervention.

Following sample preparation, the three-step PCR process is initiated.

Separating the Target DNA—Denaturation

During the first step of PCR, called denaturation, the tube containing the sample DNA is heated to more than 90 degrees Celsius (194 degrees Fahrenheit), which separates the double-stranded DNA into two separate strands. The high temperature breaks the relatively weak bonds between the nucleotides that form the DNA code.

Binding Primers to the DNA Sequence—Annealing

PCR does not copy the entire DNA in the samples. It copies only a very specific sequence of genetic code, targeted by the PCR primers. For example, Chlamydia has a unique pattern of nucleotides specific to the bacteria. The PCR will copy only the specific DNA sequences that are present in Chlamydia and absent from other bacterial species. To do this, PCR uses primers, man-made oligonucleotides (short pieces of synthetic DNA) that bind, or anneal, only to sequences on either side of the target DNA

region.Two primers are used in step two—one for each of the newly separated single DNA strands. The primers bind to the beginning of the sequence that will be copied, marking off the sequence for step three. During step two, the tube is cooled and primer binding occurs between 40 and 60 degrees Celsius (104–140 degrees Fahrenheit).Step two yields twoseparate strands of DNA, with sequences marked off by primers. The two strands are ready to be copied.

Making a Copy—Extension

In the third phase of the reaction, called extension, the temperature is increased to approximately 72 degrees Celsius (161.5 degrees Fahrenheit). Beginning at the regions marked by the primers, nucleotides in the solution are added to the annealed primers by the DNA polymerase to create a new strand of DNA complementary to each of the single template strands.After completing the extension; two identical copies of the original DNA have been made.

After making two copies of the DNA through PCR, the cycle begins again, this time using the new duplicated DNA. Each duplicate creates two new copies and after approximately 30 or 40 PCR cycles, more than one billion copies of the original DNA segment have been made. Because the PCR process is automated, it can be completed in just a few hours.

1.4.4 Phylogenetic analysis

Phylogenetics is the study of evolutionary relationships. Phylogenetic analysis is the means to infer or estimate these relationships. The evolutionary history derived from phylogenetic analysis is generally represented as branching, treelike diagrams representing an estimated pedigree of inherited relationships between molecules, organisms, or both. The phylogenetic relationship between two or more sets of sequences is often extremely important information for bioinformatic analyzes such as the construction of sequence alignment. In fact, phylogenetic relationships among many types of organisms are difficult to determine otherwise. The true relationship between homologous sequences is almost never known apart from computer simulation experiments. There are a variety of approaches available to infer the most probable phylogenetic relationship between genes and species using nucleotide and protein sequence information. Therefore, bioinformatics tends to determine the evolutionary relationship based on the statistically used inference.

Phylogenetic analysis of nucleic acid and protein sequences is currently and will continue to be an important area of sequence analysis. In addition to analyzing the changes that have occurred in the evolution of different organisms, one can study the evolution of a family of sequences. On the basis of the analysis, the sequences that are most closely related can be identified by the occupation of the neighboring branches in a tree. When a gene family is found in an organism or group of organisms, phylogenetic relationships between genes can help predict which could have an equivalent function.

1.5 Rationale

Commercial culture of tubificid worms at farmer level will sustain the production of catfish seed produced in the hatchery through reliable supply. Cultured worms will reduce the risk of outbreak of typhoid and cholera among the worm collectors caused by *Salmonela* and *Vibbrio* spp. Profiling of the amino acids of the media ingredients and the worms will provide insight into the formulation and feeding fish and worms together. Molecular identification of the worms will provide the basis for further implications of worms' classification and taxonomy.

1.6 Problem statement

Culture of tubificid worms at farmer level by using local media ingredients and no or low cost wet mediums such as cattle blood and rice gruel is not practiced elsewhere in Bangladesh. To enhance and sustain the rapid growing culture of catfishes in Bangladesh, farmed produced worms will play key role through reliable supply of good quality catfish seed by hygiene nursing and rearing.

1.7 Research needs

Ahmed and Mollah (1992) suggested 20% MOC, 35% wheat bran (WB), 25% CD and 20% sand as the suitable media for sustainable growth of tubificid worms. However, Mollah et al. (2012) have found higher production by using the mixture of 35% MOC, 20% WB, 25% CD and 20% sand. Mariom and Mollah (2012) suggested using the mixture of 20% MOC, 20% WB, 30% SBM, 20% CD and 10% sand as the best media to culture tubificid worms. Similar findings have also been confirmed by Hossain et al.(2012). Islam et al. (2015) have found highest yield of tubificid worms by using chicken blood as wet medium of the media ingredients. However, all these above

mentioned studies have been undertaken are on station and cattle blood has never been used as wet medium.

Molecular phylogeny of tubificid Oligocheates by using 16S rDNA technique has been developed by Beauchamp et al. (2001). Phylogenetic relationships of tubificidae worms have been detected by using mitochondrial 16S rDNA and nuclear 18S rDNA sequence data (Sjolin et al. 2005). Fytilis et al. (2013) distinguished tubificid taxa by assaying two hydrolysis probe-based qualitative real-time PCR (qPCR). Viven et al. (2015) found COI barcode very effective in identifying aquatic Oligocheates. Next-generation sequencing of a standard cytochrome c oxydase I (COI) barcode has been used as a rapid tool in identifying the mixed specimen samples of aquatic oligochaetes (Vivien et al. 2015). However, tubificid worms found in Bangladesh have never been identified at species level by using several molecular techniques. Therefore, there is a need to conduct off farm study at farmer level, determine the amino acid profiles of the media and worms, and identification of the tubificid worms found in Bangladesh at species level by using molecular techniques.

1.3 Objectives

The overall objective of this study is to test the possibility of commercial culture of tubificid worms at farmer level. The specific objectives are to:

i) Determine the effects of wet mediums in the yield of tubificid worms;

ii) Detect the effects of media ingredients and wet mediums on proximate and amino acid composition of tubificid worms; and

iii) Identify the tubificid wroms at species level through molecular techniques.

Chapter 2

Materials and Methods

2.1 Experimental animals and procedures

Wild harvest tubificid worms were bought from the collectors and used as the study animals. After harvest, the worms were cleaned by subsurface clean water. Before inoculation, the wild worms were conditioned by holding them in a tray with slow water flow over a period of 24 hours.

2.2 Research location

Worms were culturedat the newly constructed culture system of Maa Fatema Fish hatchery, Dalmil Jessore. Proximate composition of media ingredients were done at Institute of Nutrition and Food Science, University of Dhaka (Plate 5). Amino acid profiling of worms and media ingredients was done at BCSIR (Bangladesh Council of Scientific & Industrial Research) **(Plate 6).** Chemical composition of mud was done at Department of Soil, Water and Environment, University of Dhaka. Molecular identification of worms through COI and 16S rDNA gene were done on INVENT, Life Science Laboratory, Dhaka. The sequencing was performed in the first BASE Laboratories Sdn Bhd, Malaysia.

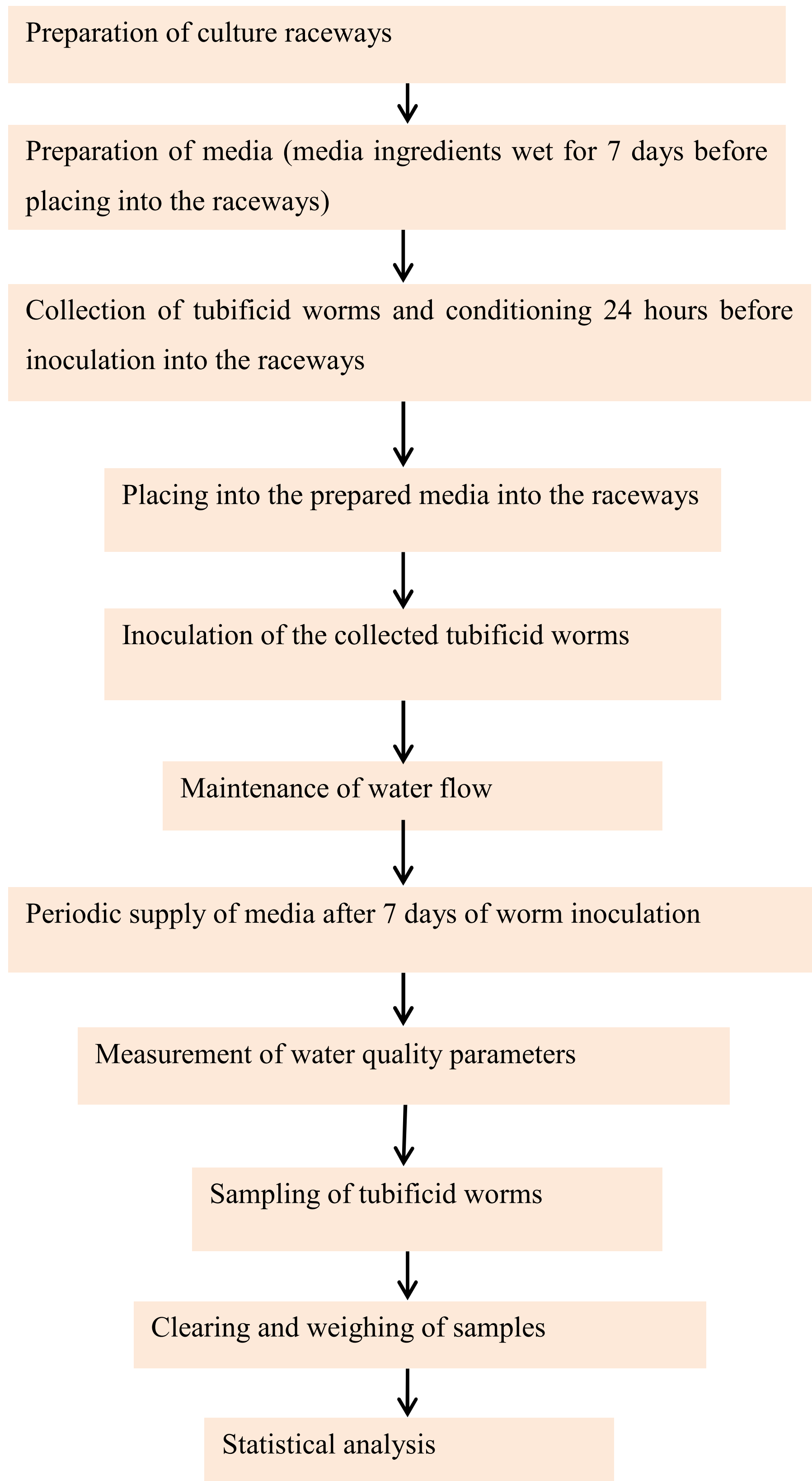
2.3 Experimental system

Newly constructed nine cemented culture raceway were (72 x 80 x 10 cm) used as culture system. Each culture unit was given continuous water flow through 1 inch diameter porous uPVC spray bar. Water flow rate was maintained to sustain dissolved oxygen concentration at 4 mg L^{-1} (Plate 3). Entire procedure of tubificid worm culture is given in **flow chart 1.**

2.4 Study design

This was a one factorial study with three replicates. Wet medium was the only experimental variable. Cattle blood, rice gruel and subsurface clean water were used as the wet mediums. Worms yield was the indicator variable. Amino acid profiles of the wild and cultured worms, media ingredients wetted in cattle blood, rice gruel and subsurface clean water was also measured.

Flow chart 1 Entire procedure of the culture of tubificid worms



2.5 Media combination

Type and quantity of media ingredients used in this study were determined through a series of experiments. Initially mixture of 20% wheat bran (WB), 30% soybean meal (SM), 20% mustard oil cake (MOC), 20% cow dung and 10% sand was tested to culture worms commercially at farmer level as suggested by Mariom and Mollah (2012) and Hossain et al (2012). However, use of CD was found to enhance fungal growth. WB did not decompose even after 7 days wetting. Therefore, WB and CD were discarded. Similarly, mud was found to enhance the growth of the worms instead of sand. Therefore, 30% SM, 40% MOC and 30% mud were used as media ingredients in the present study **(Table 1).**

Table 1 Combinations of media ingredients in three culture treatments

Ingredients (%)	Three culture treatments		
	1	2	3
Soybean meal	30	30	30
Mustard oil cake	40	40	40
Cattle blood	As required	-	-
Rice gruel	-	As required	-
Subsurface water	-	-	As required
Mud	30	30	30

2.6 Collection of media ingredients and wet mediums

Locally available SM and MOC were bought from Jessore, Bangladesh. Blood was harvested from the slaughter house and rice gruel was drawn from restaurant at free of cost. Mud was collected from nearby pond.

2.7 Media supply

Each culture unit (5760 cm^2) was given 1440 g media (250 mg cm^{-2}) upon wetting in blood, rice gruel and water for 7 days as suggested by Mariom and Mollah (2012) and Hossain et al. (2012). SBM (576 g) and MOC (432 g) were mixed and wetted in three plastic bowls with blood and held for 7 days. Similar quantities of media ingredients were also wetted in six plastic bowls with rice gruel and water. Media ingredients in each bowl were mixed well by hand twice every day. Mud was applied 1 day later of inoculation. Each culture unit was filled with water until spill out **(Plate 1, 2).**

2.8 Culture technology

Batch culture technology was applied. The system was inoculated once and the total worms were harvested after 40 days culture duration **(Plate 3, 4).**

2.9 Inoculation of the worms

The culture system was inoculated with wild harvest worms at a density of 50 mg cm^{-2} determined through a series of experiments. The worms were homogenously spread over the media in each culture unit by hand **(Plate 3).**

2.10 Renewal of the culture system

Each culture unit was renewed by 7 day wetted culture media once at 50 mg cm^2 for the first 2 weeks and twice for the next 4 weeks. Water flow was stopped before application of the media and resumed 10 minutes later.

2.11Water flow

Water flow rate was adjusted to sustain the dissolved oxygen concentration (DO) at 4 mg L^{-1}(Plate 3). Each culture unit was connected with an outlet drain. DO was measured by a portable meter (Model: HACH sension 6, Loveland, CO, USA) at 10 am.

Plate 1 Media wetting and culture system

1A Weighting media ingredients

1B Mixing with blood

1C Mixing with rice gruel

1D Mixing with water

1E Bowls with wetted media before placement

1F Media held covered after wetting

Plate 2 Media placement

2A Wetted media (blood) after 7 days

2B Wetted media (rice gruel) after 7 days

2C Wetted media (water) after 7 days

2D Media placement in the culture system

2E Spreading media into system

2F Filled with water

Plate 3 Worms' inoculation

3A Conditioning of worms

3B Measurement of dissolved oxygen

3C Weighting of worms

3D Inoculation of worms

3E Settling down of worms

3F Continuous water flow

2.12 Harvest

Entire worms from each culture unit were totally harvested by sieving. Harvested worms were cleaned by clean water **(Plate 4).** Cleaned worms were dried with blotting paper and weighted by electronic balance (SHIMADZU, Japan).

2.13 Laboratory assay

2.13.1 Proximate composition

Moisture, ash, protein, fat, fiber and available carbohydrate of the media ingredients, wetted media in blood, rice gruel and water, and cultured worms in three wet mediums **(Table 2-3)**were measured by following the method described by AOAC (2000).

2.13.2 Estimation of moisture content

2.13.2.1 Procedure

Theedible part of the each selected fruits and vegetables (10 to 11gm) was taken in a constant crucible (pre-washed and dried at 105°C). It was then kept 100-105°C temperature in an oven for 5 hours and cooled in desiccators and weighted again. Heating, cooling and weighing were continued until a constant weight was obtained.

2.13.2.2 Calculation:

$$\text{\% of moisture} = \frac{\text{Initial weight (g)} - \text{Final weight (g)}}{\text{Weight of the fresh sample (g)}} \times 100$$

Here,

Initial weight = Sample weight + crucible weight (before heating).

Final weight = Sample weight + crucible weight (after heating).

Plate 4 Worms harvesting

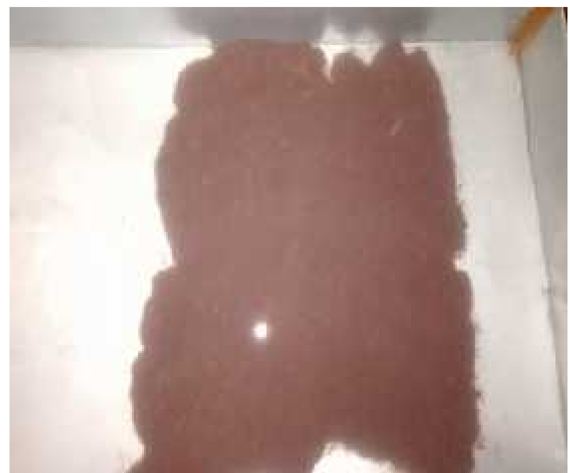
4A Before harvesting

4B Collecting worms

4C Washing

4D Migration of the worms to fine sand through mesh

4E Washing



4F Held after final wash

2.13.3 Estimation of protein content

2.13.3.1 Principle

The protein content of the food stuff is obtained by estimating the nitrogen content of the material and multiplying the nitrogen value by 6.75. This is referred to as crude protein content since the non-protein nitrogen (NPN) present in the material is not taken into consideration. True protein nitrogen can be determined by subtracting NPN from the total nitrogen.

The estimation of nitrogen is done Kjeldhal method which depends upon the H_2SO_4 with the organic nitrogen when digested in present of catalyst (selenium oxide, mercury or copper sulfate) is converted into $(NH_4)_2SO_4$. Ammonia liberated by making the solution alkaline is distilled into a known volume of a standard acid, which is then titrated.

2.13.3.1.1 Reagent preparation:

1. **Digestion mixture**

   Potassium sulphate (K_2SO_4), copper sulphate ($CuSO_4.5H_2O$) were powdered with mortar and pestle and mixed well in a ratio of 98 g: 2 g.

2. **Sodium hydroxide solution (40%)**

   Sodium hydroxide (4gm) was dissolved in distilled water and volume was made up to 100ml.

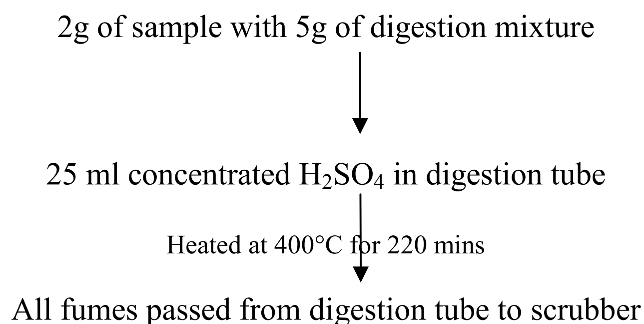
3. 0.1 N H_2SO_4
4. 0.1 N Na_2CO_3
5. 0.1 N NaOH
6. Concentrated H_2SO_4
7. Distilled water
8. Methyl red indicator (3-4 drops)

2.13.3.1.2 Procedure

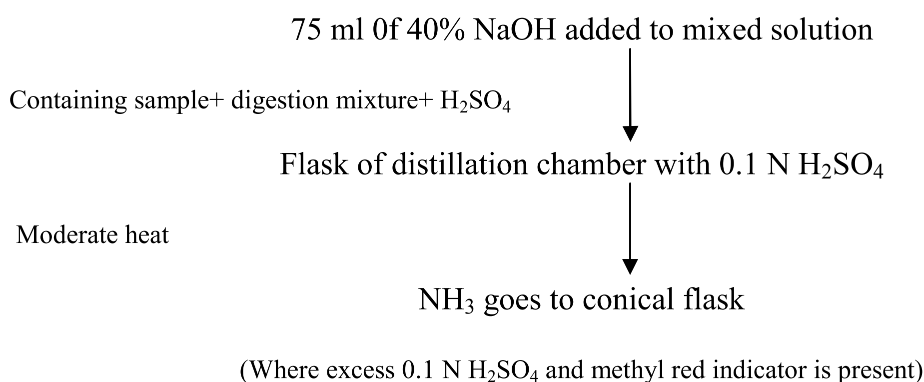
The Kjeldhal method consists of the following steps:

1. Digestion of the sample
2. Distillation
3. Titration

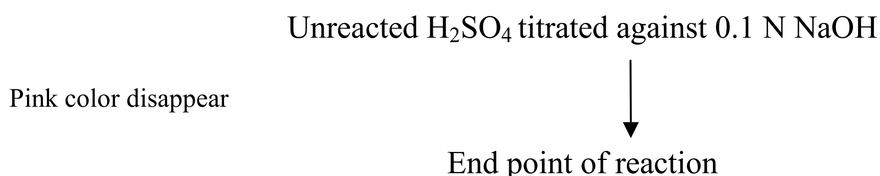
Digestion



Distillation



Titration



2.13.3.1.3 Calculation

The protein content of sample on the percentage basis was calculated by the following formula.

$$\% \text{ of protein} = \frac{(c-b) \times 14 \times d \times 6.25 \times 100}{a \times 1000}$$

Where,

a = sample weight (g)

b = volume of NaOH required for the back titration and to neutralize 20 ml of 0.1 N H_2SO_4 (for sample).

c = volume of NaOH required for the back titration and to neutralize 20 ml of 0.1 N

NH_2SO_4 (for blank)

d = normality of NaOH used for titration.

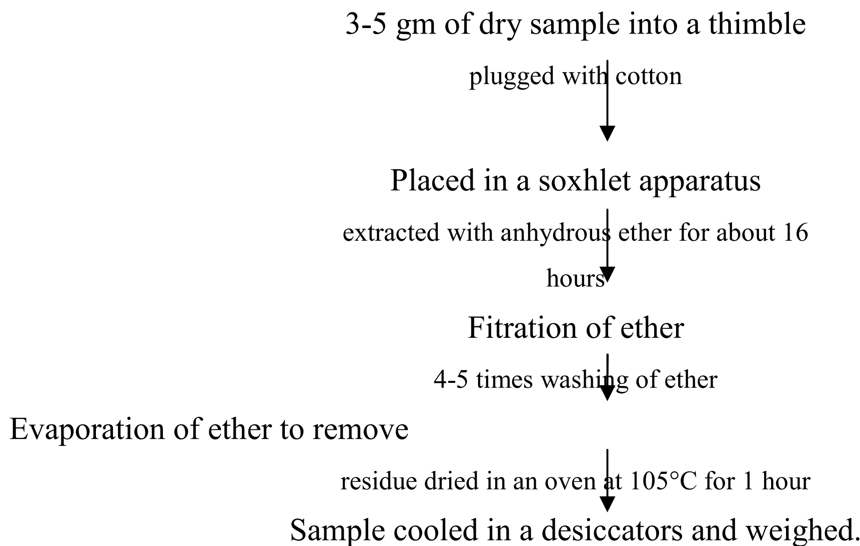
The conversion factor of nitrogen to protein is 6.25 and atomic weight of nitrogen is 14.

2.13.4 Estimation of fat content

2.13.4.1 Principle

Fat is estimated as crude extract of the dry material (by soxhlet method). Here a suitable solvent of petroleum ether can be used. Fat is estimated by dissolving the food sample into organic solvent (petroleum ether) separating the filtrate by filtration. Placing the filtrate into the separating funnels and then separated mixture is dried to measure the extract and finally the percentage of the fat was calculated.

2.13.4.2 Procedure



2.13.4.3 Calculation

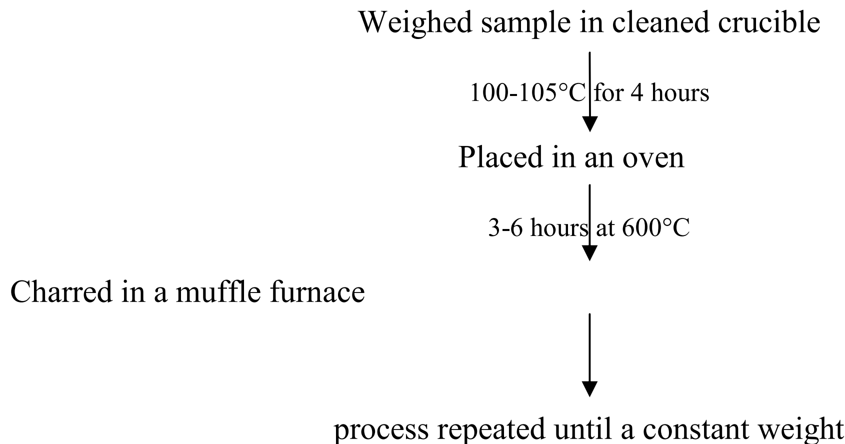
$$\% \text{ of fat} = \frac{\text{Weight of the extract}}{\text{Weight of the sample taken}} \times 100$$

2.13.5 Estimation of ash content

2.13.5.1 Principle

The ash content of the food stuff is determined by drying the food at high temperature. Ash indicates the total amount of mineral in the food sample. It is the inorganic compound.

2.13.5.2 Procedure



2.13.5.3 Calculation

$$\% \text{ of ash} = \frac{\text{Weight of ash (g)}}{\text{Weight of sample (g)}} \times 100.$$

2.13.6 Estimation of TDF (total dietary fiber)

2.13.6.1 Principle

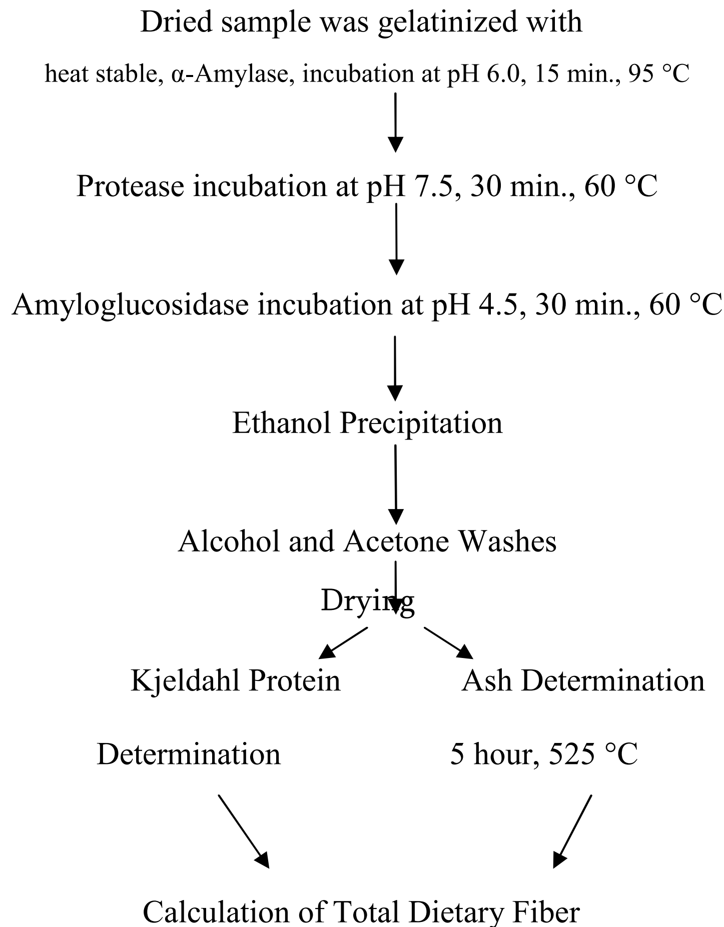
Dietary fiber is generally defined as lignin plus plant polysaccharide that can't be digested by human enzyme. Dietary fiber is estimate by two basic approaches gravimetrically or chemically. In gravimetric method digestible carbohydrate, lipids and proteins are selectively solubilized by chemicals or enzymes. Indigestible materials are then collected by filtration, and the fiber residue is calculated gravimetrically (by weightdifference). Four enzyme systems (salivary and pancreatic amylase, lipase, protease and glucose-oxidase) are used for fiber estimation.

2.13.6.2 Reagents:

1. Pancreatin powder contains Alpha (α) amylase, protease and lipase enzyme
2. Enzyme glucose-oxidase
3. Tris buffer(Hydroxy methyl amino methane)

4. Celite particle
5. Alcohol
6. Acetone

2.13.6.3 Procedure



2.13.6.4 Calculation

$$\% \text{ of total dietary fiber (TDF)} = \frac{\text{Weight of residue (g)}}{\text{Weight of sample (g)}} \times 100$$

2.13.7 Calculation of Carbohydrate

The content of available carbohydrate in the food sample was determined by difference. Carbohydrate was calculated by subtracting the sum percentage of moisture, protein, fat, ash, crude and dietary fiber. The nitrogen free extract (NFE) was obtained by subtracting the sum of the values for moisture, protein, fat and ash from 100 (Ferris et.al. 1995). This value was considered as "total carbohydrate" and was calculated by following equation.

Carbohydrate (NFE g %) = 100 − (Protein+ lipid + moisture +ash + TDF) g/100 g.

Table 2 Nutrient composition of the media ingredients and wetted media in blood, rice gruel and water

Composition (Dry matter basis)	Ingredients			
	Soybean meal	Blood	Mustard oil cake	Rice gruel
Protein (%)	49.15	81.83	35.11	7.89
Fat (%)	5.76	1.68	15.11	4.45
Ash (%)	7.50	4.14	11.24	3.38
Fiber (%)	1.0	1.0	1.0	1.45
Carbohydrate (%)	36.58	11.93	37.56	81.38
Total	100	100	100	100
Calorie (Kcal/100 g)	361.11	157.18	375.21	773.11

Table 3 Nutrient composition of the media ingredients wetted in blood, rice gruel and water sampled after 7 days wetting

Composition (Dry matter basis)	Mediums used to wet the media ingredients		
	Cattle blood	Rice gruel	Subsurface water
Protein (%)	58.16	46.74	43.67
Fat (%)	13.43	15.43	17.60
Ash (%)	8.27	8.71	2.90
Fiber (%)	1.0	1	1.0
Available carbohydrate (%)	19.14	28.12	34.83
Total	100	100	100
Calorie(Kcal/100 g)	124.63	127.12	116.90

2.13.8Amino acid profiling

Amino acid profiles of the media ingredients wetted in water, rice gruel and cattle blood and wild and cultured worms were determined by an automatic Amino Acid Analyzer (HitachiL-8800, Tokyo, Japan). Some 0.5 g isolated protein was pasted with 50 ml 6 N HCl by mortar pestle, by placing in heating plate at 110 C for a period of 24 h, filtered through Whatman No. 9 filter paper. The filtrate was hydrolyzed over 22–24 h ina

hydrolysis tube. After hydrolyzing, HCl was removedfrom the filtrate by evaporating in water bath. After evaporation,the solution was volume to 25 ml in volumetric flask by0.1 N HCl and run through Amino Acid Analyzer.

Table 4Amino acid profiles of the media ingredients wetted in blood, rice gruel and water sampled after 7 days wetting

Aminoacid (% protein)	**Media ingredients wetted in**		
	Water	Rice gruel	Blood
Essential amino acids			
Threonine	8.7	10.6	12.2
Leucine	0.88	1.13	1.15
Methionine	2.33	3.3	3.58
Lysine	4.56	4.92	5.12
Arginine	2.49	2.66	2.85
Valine	0.81	0.93	1.06
Isoleucine	2.8	3.3	3.93
Histidine	0.59	0.68	0.74
Non-essential amino acids			
Aspartic acid	4.72	5.36	6.24
Serine	2.3	2.69	2.85
Glutamic acid	2.32	3.61	3.75
Glycine	2.36	2.53	2.67
Alanine	2.37	2.7	2.99
Tyrosine	1.5	1.74	1.84

2.13.9 Chemical Composition of Mud

Mud chemical composition was also analyzed following the method described by Huq and Alam (2005).

Plate 5 Determination of protein

5A Weighing (Sample)

5B Weighing (40% NaOH)

5C mixing with digestion mixer

5D Sample in distillation chamber

5E Unreacted H_2SO_4 titrated against NaOH

5BPink color disappear

Plate 6 Amino acid profiling

6A samples (media ingredients after wetting)

6B sample preparation with 6N HCl for amino acid profile

6C Evaporation

6D Sample in the heating mental for hydrolysis

6E Sample passed through microfilter

6F Sample in amino acid analyzer

2.14 Molecular identification (Flow chart 2)

2.14.1 Automated DNA Extraction

In the automated DNA extraction method 50 mg of mixed worms sample was loaded into well#1 of the Maxwell® 16 SEV DNA Cartridge. Plunger was set at well#7 of the Maxwell® 16 SEV DNA Cartridge. The Maxwell® 16 DNA Cartridge contains an array of chamber facilities for DNA extraction. About 300 µl of elution buffer was loaded in the elution tube. After that The Maxwell® 16 SEV DNA Cartridge and elution tube were placed in the specified chamber of the instrument. DNA extraction was carried out for 46 minutes.

2.14.2 Measurement of the Concentration of Extracted DNA

The concentration and purity of the extracted DNA were measured by NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). The product was measured as ng/µl. The reading of the ratio was between at 260 nm and 280 nm (OD 260 / OD 280). This OD ratio provides an estimate the purity of nucleic acid (DNA) which is a value of 1.8.

2.14.3 Conventional PCR Amplification

The extracted DNA was directly amplified by adding GoTaq® Hot Start Colorless Master Mix (Promega, USA). Composition of the GoTaq® Hot Start Colorless Master Mix is given in **Table 5.** Initial screening was done with two set of primers **(Table 6)**.

Table 5 Composition of GoTaq® Hot Start Colorless Master Mix

GoTaq® Hot Start Colorless Master Mix (2)
GoTaq® Hot Start Polymerase
dNTPs (400µM each)
2× Colorless GoTaq® Reaction Buffer (pH 8.5)
$MgCl_2$(4 mM)

Flow chart 2 Molecular identification of tubificid worms

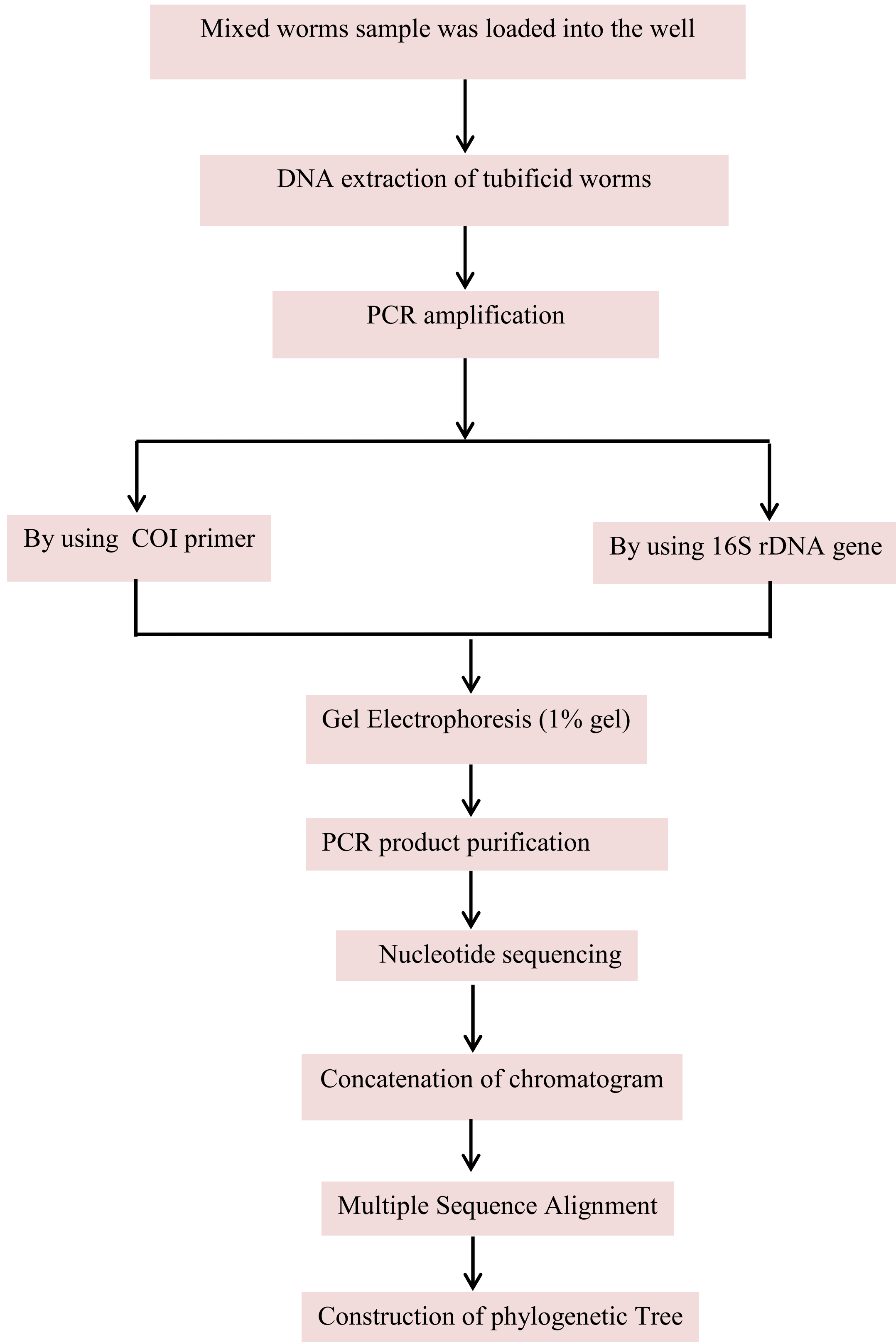


Table 6 Primer sequences used for conventional PCR amplification

Primer	Sequence (5´-3´)	References
LCO 1490	5´ GGT CAA CAA ATC ATA AAG ATA TTG G 3´	Folmer et al., 1994
HCO 2198	5´ TAA ACT TCA GGG TGA CCA AAA AAT CA 3´	
16 Sar	5´CGC CTG TTT ATC AAA AAC AT 3´	Palumbi et al., 1991
16 Sbr	5´ CCG GTY TGA ACT CAG ATC AYG T3´	

PCR mix was prepared with the addition of 2×GoTaq® Hot Start Colorless Master Mix, 400nM of each of the primers and nuclease free water. Although GoTaq® Hot Start Polymerase component of the master mix was bound to a proprietary antibody that blocks polymerase activity at temperature below 70°C, PCR mix was prepared maintaining ice-cold condition. After mixing the mixture by vortex, short centrifugation was done. After that, the reaction mix was dispensed into sterile, thin walled PCR tubes. Both positive and negative control reactions were performed to authenticate the PCR **(Table 7)**.

All the PCR tubes containing the appropriate mixtures were heated at 95 ºC for 4 minutes in the thermal cycler (Applied Biosystem, USA) to ensure denaturation of all DNA templates. Thirty (30) cycles of these segments were repeated with a final extension of 5 minutes at 72ºC. After this, PCR tubes were stored at -20 ºC until further analysis. The cycling profile for each primer: target combination was optimized accordingly **(Table 8).**

2.14.4 Agarose Gel Electrophoresis

The amplified products were run on 1 % agarose gel with a 100 bb-DNA ladder (Promega, USA) for visualization of the amplified products. Agarose (Agarose LE, Analytical grade, Promega, Spain) was measured at an amount of 0.60 g and mixed with 60 ml 1x TAE buffer **(Appendix H)** to prepare 1% agarose gel (Appendix H). The mixture was heated in microwave for ~5 min on medium until the agarose melted. Then the boiled mixture was allowed to cool to about 45°C before 3µl Ethidium bromide (stock 10 mg/ml) was added. The gel was poured onto gel casing and well former

(comb)was inserted. The casing was then allowed to set on a flat surface for about 15 min. 1x TAE Buffer was then poured into the electrophoresis tank and comb was removed from gel. Samples were mixed with loading dye on parafilm (1µl loading buffer and 5µl PCR product). Molecular weight marker was prepared by mixing 6µl molecular weight marker and 1µl loading buffer. Samples were loaded into the wells formed in the gel using sterile tips. Electrophoresis was set at 100 volts for 45 min. The gel was viewed on AlphaImager HP Gel-documentation system (Cell Bioscience, USA).

Table 7 Preparation for Conventional PCR

PCR Mixture Components	Positive Control (50µl reaction)	Negative Control (50 µl reaction)	Experimental Reaction (50 µl reaction)
Nuclease Free Water	Up to 50 µl	Up to 50 µl	Up to 50 µl
GoTaq® Hot Start Colorless Master Mix (2×)	25	25	25
Upstream Primer (10 µM)	2 µl (400nM)	2 µl (400nM)	2 µl (400nM)
Downstream Primer (10 µM)	2 µl (400nM)	2 µl (400nM)	2 µl (400nM)
Template	Variable (< 500 ng)	No Template	Variable (< 500 ng)

Table 8 Temperature and time profile of the thermocycler

Steps	Temperature and Time Profile
Denaturation	95 ºC for 60 seconds
Annealing	48 ºC for 30 seconds
Extension	72 ºC for 45 seconds

2.14.5 PCR Purification

2.14.5.1 Purification of Amplicon

After Agarose gel electrophoresis the PCR positive sample was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA; Appendix H). Centrifugation based methodology was followed. The Wizard® SV Gel and PCR Clean-Up System are based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts (guanidine isothiocyanate). After amplification, an aliquot of the PCR was added to the guanidine isothiocyanate containing Membrane Binding Solution (MBS) and directly purified. An equal volume of MBS was added to PCR amplification. The mixture was transferred to the mini column pre-set with a collection tube (SV mini column assembly). After short (2 minutes) incubation at room temperature, SV mini column was centrifuged at 16,000×g (14,000rpm) for 1 minute. After discarding the flow-through, SV mini column was subjected to wash for two times with Membrane Wash Solution (Supplied in the kit,ethanol added). After washing the SV mini column, DNA was eluted in Nuclease Free Water (Supplied in the kit). The purified plasmid was stored at -20°C until further processing.

2.14.6 Measurement of the Concentration of the Amplicon

The amount of PCR product was measured according to the protocol described in chapter **2.14.2.**

2.14.7 Sequencing Based Identification

2.14.7.1 Sequencing Reaction

For confirmation of the PCR products, cycle sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, USA) according to the manufacturer's instruction. Extension product was purified followed by capillary electrophoresis using ABI Genetic Analyzer (Applied Biosystems®, USA). The basic sequencing protocol is illustrated below.

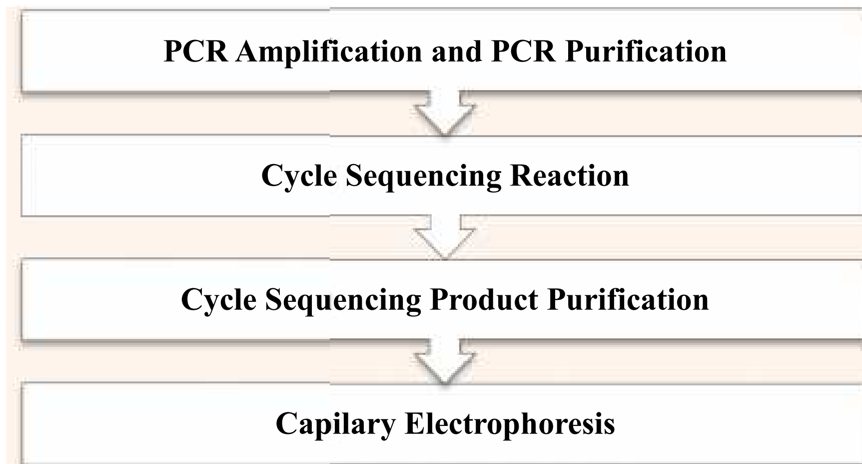


Figure 4 Sequencing of PCR products of Tubificid worms

2.14.7.2 Sequence Alignment and Bioinformatics analysis

Partial sequences of desired genes obtained using specific forward and reverse primers were combined to full length sequences using the SeqMan Genome Assemblerand were compared to the GenBank database of the National Center for Biotechnology Information (NCBI) by means of the basic local alignment search tool (BLAST) to identify their close phylogenetic relatives.

2.14.7.3 Bioinformatics tools

Different bioinformatics tools were used to analyze the sequences. These tools are given below

NCBI BLAST

BLAST is a toolkit developed by the National Center for Biotechnology Information (NCBI), the US-based organization responsible for archiving and databasing the world's genetic sequence information.BLAST for the basic local alignment search tool is an algorithm for comparing primary biological sequence information, such as amino acid sequences of proteins or nucleotides of DNA sequences. A BLAST search allows an investigator to compare a sequence of queries with a library or database sequences and identify sequences of libraries that resemble the query sequence above a certain threshold.

Finch TV VERSION 1.4

FinchTV (Finch Trace Viewer), a cross-platform graphical viewer for chromatogram files. It is used to read the chromatogram files of the most popular formats. Simply drag and drop AB1 or SCF files, even compressed with gzip, into the display window to

showa multi-colored sequence. FinchTV also displays quality values, when available, and can scale both directions vertically and horizontally in both single and multi-view views. FinchTV can print the data, edit the data, export FASTA sequences and view all the details stored in sequence files.

Figure 5 Chromatogram of the forward (F) and reverse (R) sequence of isolate TD_Tubifex_COI in FinchTV.

DNASTAR SeqMan

SeqMan NGen is a program that has the ability to assemble any size genome quickly and accurately on a desktop computer. It allows you to run and manage NGS assemblies directly on the Cloud using DNASTAR Cloud Assemblies. SeqMan NGen assembles data from all major next-gen sequencing platforms, and provides an extremely easy-to-use interface that steps you through your sequence assembly and analysis project.

CLUSTALW

CLUSTALW produces sequences of biologically significant multiple sequences of divergent sequences. It calculates the best match for selected sequences and lists them, so that identities, similarities and differences can be identified.

Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 software

The Molecular Evolutionary Genetics Analysis (MEGA) software is developed for comparative analyses of DNA and protein sequences that are aimed at inferring the molecular evolutionary patterns of genes, genomes, and species over time (Tamura et al. 2011). MEGA is currently distributed in two editions: a graphical user interface (GUI) edition with visual tools for exploration of data and analysis results (Tamura et al. 2011) and a command line edition (MEGA-CC), which is optimized for iterative and integrated pipeline analyses (Kumar et al. 2012). In version 6.0, facilities are added for building molecular evolutionary trees scaled to time (timetrees), which are clearly needed by scientists as an increasing number of studies are reporting divergence times for species, strains, and duplicated genes. In the RelTime method, which can be used for large numbers of sequences comprising contemporary data sets, is the fastest method among its peers, and is shown to perform well in computer simulations (Tamura et al. 2012). RelTime produces estimates of relative times of divergence for all branching points (nodes) in any phylogenetic tree without requiring knowledge of the distribution of the lineage rate variation and without using clock calibrations and associated distributions.

2.14.8 Phylogenetic Analysis

2.14.8.1 Construction of Phylogenetic tree

Phylogenetic tree of the COI and 16S rDNA gene sequences of Tubificid worms was constructed using the following servers and bioinformatics software:

A. Reference sequences were downloaded from

NCBI: http://www.ncbi.nlm.nih.gov

B. Partial sequences, obtained using forward and reverse primers, were combined to full length sequences via the SeqMan Genome Assembler and then aligned, checked and processed by using Molecular Evolutionary Genetics Analysis (MEGA) version 6 (Tamura et al., 2013), an integrated tool for conducting sequence alignment, inferring phylogenetic trees, estimating divergence times, mining online databases, estimating

rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses.

C. Phylogenetic tree was constructed by MEGA 6.

Briefly, the multiple sequence alignment of the retrieved reference sequences from NCBI and sequences of the amplified gene was performed with the ClustalW program embedded in Mega 6. Aligned sequences were refined by sequence trimming and conserved region identification. Refined sequences were used for selecting best model and phylogenetic tree construction using the Neighbor-Joining Algorithm and selecting 1000 bootstrap replication. Further analysis of the genes was carried out using the Distance and Pattern analysis tools in the MEGA software.

2.15 Data analysis

All percent data were transformed into square root before analysis. Data were analyzed by 1-way anova followed by Tukey's HSD post hoc for multiple comparisons. SPSS version 20.0 was used to analyze the data. Level of significance considered in this study in analyzing the data was $p<0.05$.

Chapter 3
Results

3.1 Yield of tubificid worms

The highest yield of tubificid worms (683.68 ± 3.86 mg cm^{-2}) was harvested from the treatment where media ingredients were wetted in cattle blood while the lowest yield (584. 38 ± 1.41 mg cm^{-2}) was found in the treatment in which the media ingredients were wetted in subsurface clean water (**Figure 6, Appendix A-C**).Rice gruel wetted media resulted in the yield of worms (615.63 ± 3.66 mg cm^{-2}) that was significantly different from other two treatments.

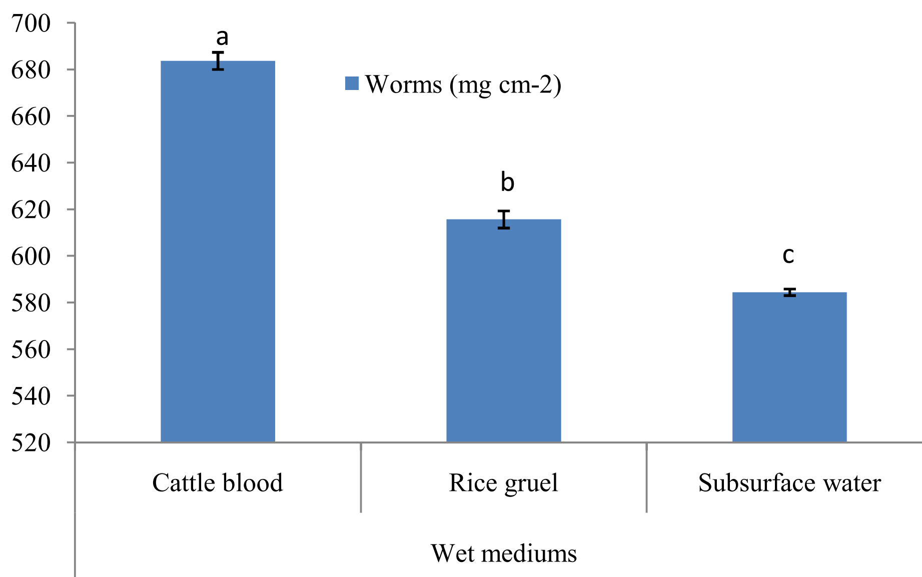


Figure 6 Yield of tubificid worms (mg cm^{-2}) harvested from the media wetted by cattle blood, rice gruel and subsurface water after 40 day culture duration in cemented culture raceway of 0.567 m^2 at farmer level. Bars (mean ± 1 SEM) with different letters indicate significant differences (ANOVA, HSD; $p<0.05$).

3.2 Proximate composition of the worms

Worms raised in the media wetted in blood had nearly 58% protein followed by rice gruel (55%) and water (53%) **(Table 9).** Similarly highest level of fat (13%) was detected in the worms produced in the media wetted in blood followed by rice gruel (12%) and water (11%). Similarly highest level of Calorie (Kcal/100 g) was measured in the worms raised in the media wetted in rice gruel (85) followed by blood (79) and water (68).

Table 9 Proximate composition of worms

Composition (%)	Worms produced in the culture media wetted in		
(Dry matter basis)	Blood	Rice gruel	Water
Protein	58.2	55.5	53.24
Fat	13.2	11.68	11.04
Ash	5.02	3.50	5.07
Fiber	4.24	3.31	3.29
Carbohydrate	19.34	26.01	27.36
Total	100	100	100
Calorie (Kcal/100 g)	79.23	85.09	68.03

3.3 Amino acid profiles of the wild and cultured worms

Total 14 amino acids were detected due to the hydrolysis of the sample in HCl. All 14 amino acids were found at significantly higher level in the worms raised in the media wetted in blood than in the wild, rice gruel and water **(Table 10, Appendix D-F)**. Of 8 essential amino acids (EAA) detected in the worms, lysine, arginine and leucine were found highest level in the worms raised in the media ingredients wetted in blood followed by water and rice gruel. Among non-essential amino acids (NEAA), worms raised in the media wetted in blood had the highest level of glycine followed by wild worms. Level of glycine was similar in the worms raised in the media wetted in rice gruel. Alanine and glutamic acid followed similar trends in occurrence.

Table 10 Amino acid profiles of the wild and cultured worms in which media ingredients were wetted in blood, rice gruel and subsurface clean water

Amino acids (% of protein)	Types of worms raised in the culture media wetted in			
	Wild	Blood	Rice gruel	Water
Essential amino acids				
Threonine	$1.7 \pm 0.01_d$	$3.62 \pm 0.03_a$	$2.71 \pm 0.02_b$	$2.60 \pm 0.02_c$
Leucine	$4.50 \pm 0.12_b$	$5.19 \pm 0.21_a$	$4.20 \pm 0.08_c$	$3.80 \pm 0.07_d$
Methionine	$2.70 \pm 0.01_b$	$3.40 \pm 0.04_a$	$2.50 \pm 0.01_c$	$2.30 \pm 0.02_d$
Lysine	$6.41 \pm 0.41_b$	$6.87 \pm 0.34_a$	$5.80 \pm 0.53_c$	$5.60 \pm 0.06_d$
Arginine	$5.80 \pm 0.06_b$	$6.40 \pm 0.08_a$	$5.38 \pm 0.05_c$	$4.77 \pm 0.07_d$
Valine	$2.60 \pm 0.01_b$	$3.70 \pm 0.04_a$	$2.20 \pm 0.01_c$	$2.20 \pm 0.01_c$
Isoleucine	$2.50 \pm 0.01_c$	$4.50 \pm 0.13_a$	$2.80 \pm 0.03_b$	$2.51 \pm 0.01_c$
Histidine	$1.80 \pm 0.02_b$	$2.41 \pm 0.04_a$	$1.60 \pm 0.03_c$	$1.50 \pm 0.01_d$
Non-essential amino acids				
Aspartic acid	$3.51 \pm 0.02_b$	$4.73 \pm 0.05_a$	$3.56 \pm 0.05_b$	$3.38 \pm 0.02_b$
Serine	$3.07 \pm 0.05_b$	$4.20 \pm 0.01_a$	$3.21 \pm 0.01_b$	$3.04 \pm 0.05_b$
Glutamic acid	$4.91 \pm 0.02_b$	$5.91 \pm 0.02_a$	$4.80 \pm 0.01_c$	$4.50 \pm 0.01_d$
Glycine	$6.13 \pm 0.03_b$	$7.03 \pm 0.20_a$	$5.40 \pm 0.01_c$	$5.30 \pm 0.01_c$
Alanine	$4.70 \pm 0.01_c$	$5.80 \pm 0.13_a$	$4.80 \pm 0.11_b$	$4.60 \pm 0.10_d$
Tyrosine	$2.50 \pm 0.02_b$	$3.61 \pm 0.06_a$	$2.40 \pm 0.02_b$	$2.37 \pm 0.26_b$

3.4 Chemical composition of mud

Mud contained 0.51% nitrogen, 52.70 ppm phosphorus and 112.28 ppm potassium.

3.5 Molecular identification of Tubificid worms

The final concentration and purity of the 3 sample are given in **Table 11.**

Table 11 Measurement of DNA concentration of 3 samples

Sample ID	Concentration of DNA (ng/µl)	Purity (260/280)
TD_Tubifex	21.9	1.93
TM_Tubifex	104.2	1.88
TJ_Tubifex	7.5	1.82

3.5.1 COI sequence based identification

3.5.1.1 PCR amplification

The extracted DNA from tubificid worms of three different region (Dhaka denoted by TD, Mymensingh indicated by TM, Jessore denoted by TJ) was amplified by PCR amplification for COI gene using COI specific primer LCO1490 and HCO2198, which has product size of around 710 bp. The representative gel autoradiograph of the amplicon is showed in **Figure 7**.

3.5.1.2 Sequence output

From the chromatogram, the sequence data were transferred to fasta format and blasted within nucleotide database to identify species. 3 samples were sequenced and blasted. The mitochondrial cytochrome c oxidase subunit 1 (COI) region of all samples was successfully amplified using PCR. The comprehensive identification results were based on Genbank databases. Database revealed that identity matches in the range of 81-87% for consensus sequences of 3 sample tubifex species. Genbank based identification showed that species alignment E value is 0.0 for TD_Tubifex_COI and TJ_Tubifex_COI and 6E-139 for TM_Tubifex_COI.

Table 12 COI sequence based identification of representative tubificid worms from three different region

Sample ID	Identified species	Max score	Total score	Query cover	E value	Identity	Accession
TD_Tubifex_COI	*Tubifex tubifex*	713	713	99%	0.0	87%	HM138065.1
TM_Tubifex_COI	*Tubifex tubifex*	505	505	100%	6e-139	81%	EU311345.1
TJ_Tubifex_COI	*Branchiura sowerbyi*	726	726	100%	0.0	87%	LN810299.1

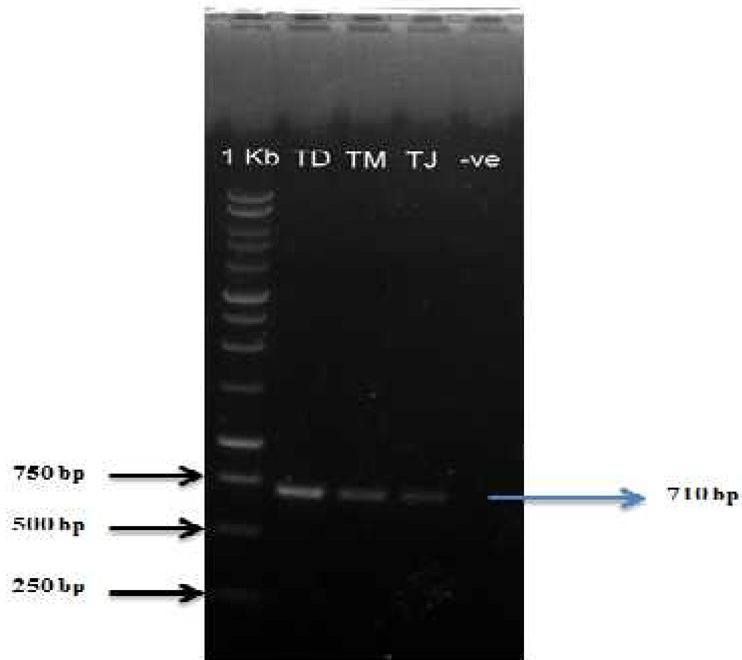
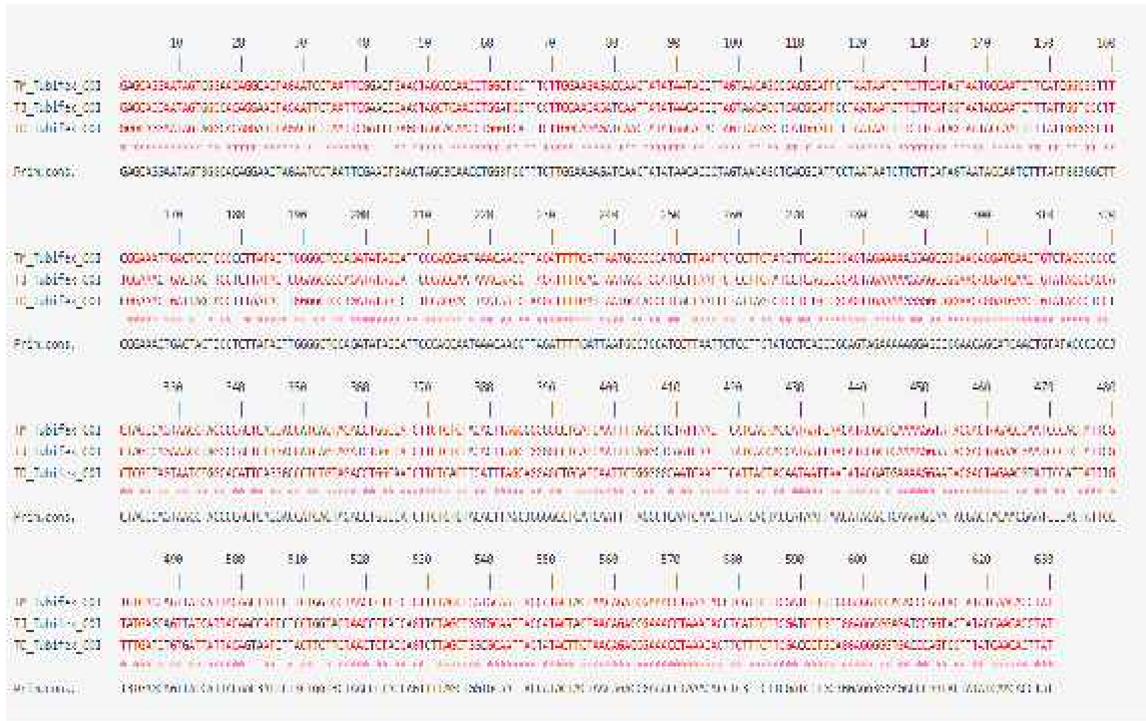


Figure 7 PCR amplification product profiles of COI primer generated from 3 different Samples: TD,TM,TJ. Amplicon size was 710 bp. Here, 1 kb marker was used. Sample ID TD, TD_Tubifex_COI; TM, TM_Tubifex_COI; TJ, TJ_Tubifex_COI.

Legend: (-ve) denotes negative control.

3.5.1.3 DNA sequence alignment

3.5.1.3.1 Alignment among Sample TD_Tubifex_COI, TM_Tubifex_COI and TJ_Tubifex_COI



Alignment Data

Alignment length 630

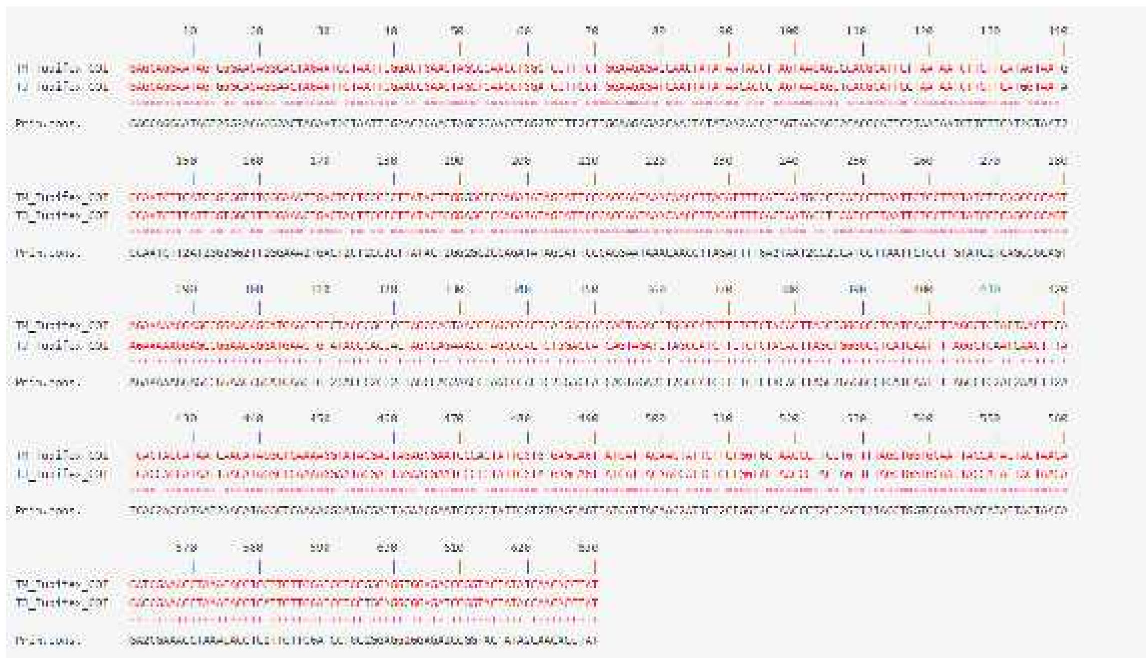
Identity(*): 476 is 75.56%

Different: 154 is 24.44%

Figure 8 Multiple sequence alignment of COI gene fragment of three Tubifex species TD_Tubifex_COI, TM_Tubifex_COI, TJ_Tubifex_COI where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 160)

Multiple sequence analysis is shown by view CLUSTALW to compare the sequences. After comparing the obtained sequence of 3 sample of tubificid worms, total 154 sites were found polymorphic. Therefore the dissimilarity was 24.22%. Among 3 sequences TJ_Tubifex_COI (cultured) was closely related to TM_Tubifex_COI while less relation was found with TJ_Tubifex_COI and TD_Tubifex_COI.

3.5.1.3.2 Alignment between TM_Tubifex_COI and TJ_Tubifex_COI



Alignment Data

Alignment length 630

Identity(*): 568 is 90.16%

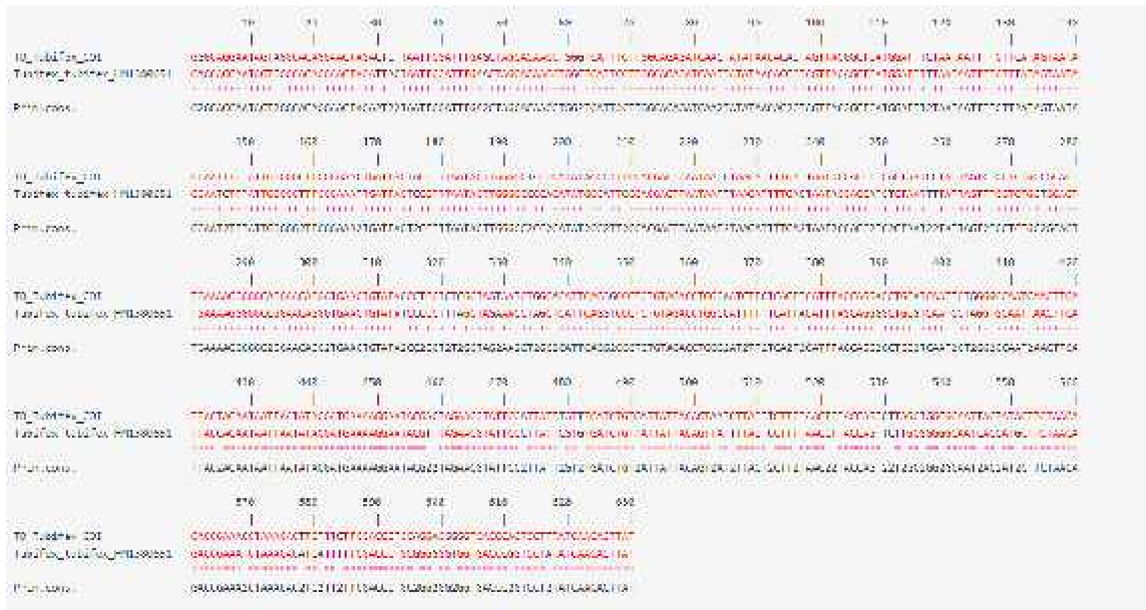
Different: 62 is 9.84%

Figure 9 Pairwise sequence alignment of COI gene fragment of closely related species TM_Tubifex_COI and TJ_Tubifex_COI where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 140)

Table 13 Polymorphic sites observed between closely related species TM_Tubifex_COI and TJ_Tubifex_COI

Position	TM_Tubifex_COI	TJ_Tubifex_COI
14	C	G
41	G	A
140	G	A
191	G	A
320	C	A
419	C	T
551	G	A
581	G	A

3.5.1.3.3 Alignment between TD_Tubifex_COI and *Tubifex tubifex*



Alignment Data

Alignment length 630

Identity(*): 547 is 86.83%

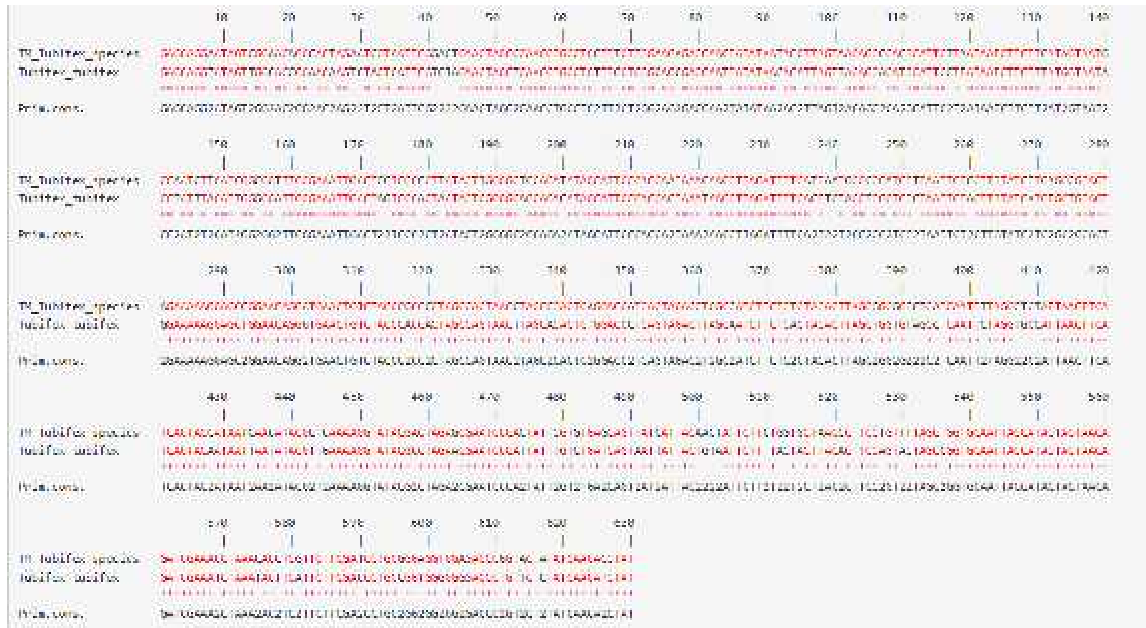
Different: 83 is 13.17%

Figure 10 Pairwise sequence alignment of COI gene fragment of sample TD_Tubifex_COI and GenBank reference *Tubifex tubifex* where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 140)

Table 14 Polymorphic sites observed between TD_Tubifex_COI and *Tubifex tubifex*

Position	TD_Tubifex_COI	*Tubifex tubifex*
14	A	G
62	G	C
131	C	T
242	G	A
293	A	C
371	C	T
473	A	C
551	A	G
617	T	A

3.5.1.3.4 Alignment between TM_Tubifex_species and *Tubifextubifex*



Alignment Data

Alignment length 630

Identity(*): 510 is 80.95%

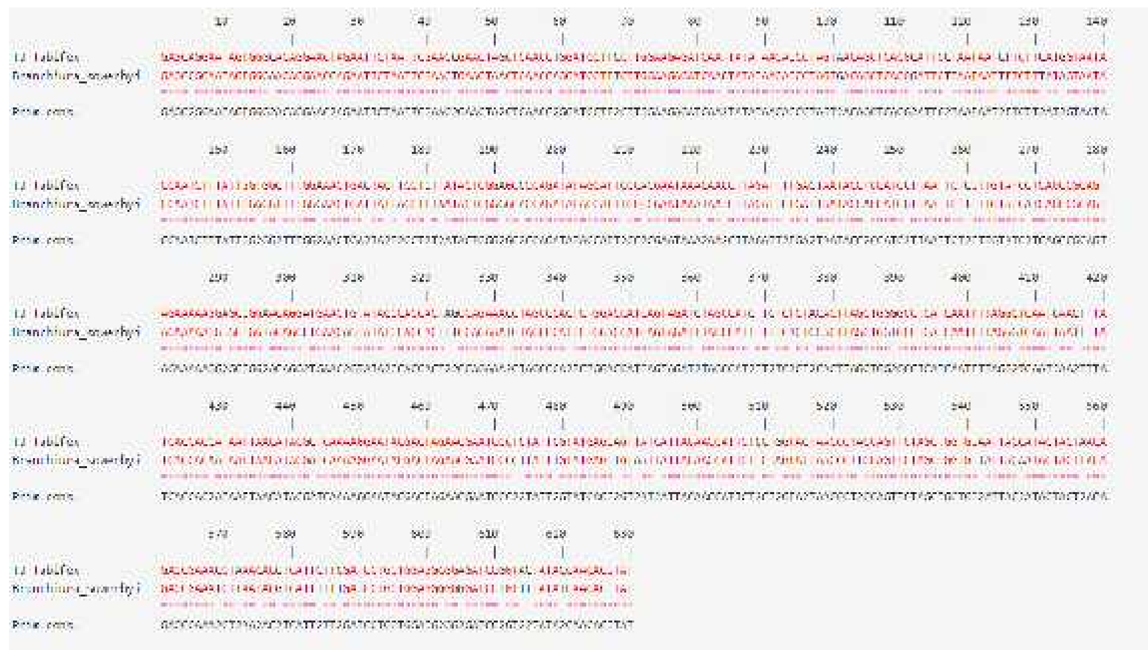
Different: 120 is 19.05%

Figure 11 Pairwise sequence alignment of COI gene fragment of sample TM_Tubifex_COI and GenBank reference *Tubifex tubifex* where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 140)

Table 15 Polymorphic sites observed between TM_Tubifex_COI and *Tubifex tubifex*

Position	TD_Tubifex_COI	*Tubifex tubifex*
8	A	T
53	C	T
140	G	A
221	C	T
332	A	G
360	C	T
482	G	C
569	C	T
611	G	T

3.5.1.3.5 Alignment between TJ_Tubifex_species and *Branchiura sowerbyi*



Alignment Data

Alignment length 630

Identity(*): 551 is 87.46%

Different: 79 is 12.54%

Figure 12 Pairwise sequence alignment of COI gene fragment of sample TJ_Tubifex_COI and GenBank reference *Branchiura sowerbyi* where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 140)

Table 16 Polymorphic sites observed between TJ_Tubifex_COI and *Branchiura sowerbyi*

Position	TJ_Tubifex_COI	*Branchiura sowerbyi*
17	C	A
131	C	T
171	C	T
269	C	A
341	C	T
443	C	A
512	G	A
572	A	T
620	C	T

3.5.1.4 Phylogentic analysis

Multiple sequence analysis was done using examined sequences and downloaded (Table 17) sequences and phylogenetic tree was constructed. Phylogenetic analysis based on COI gene sequences of the extracted DNA from 3 sample tubificid worms using neighbor joining confirmed the taxonomic position of the worms. From the tree it is clearly showed that TM_Tubifex_COI and TJ_Tubifex_COI is closely related with *Branchiura sowerbyi* and TD_Tubifex_COI is closely related with Tubifex tubifex and *Limnodrilus hoffmeisteri.*

Table 17 List of all downloaded species from NCBI GenBank to support the phylogenetic analysis

Downloaded species	GenBank Accession no
Tubifex tubifex	HM138065.1
Limnodrilus hoffmeisteri	EU311398.1
Amynthas cortices	KF205966.1
Branchiura sowerbyi	AF534864.1
Limnodrilus udekemianus	LN810320.1
Tubifex ignotus	GU902114.1
Riftia_pachyptila	AY645991.1
Potamothrix bavaricus	LN810330.1
Slavina appendiculata	GQ355375.1
Eisenia eiseni	AY874487.1
Hormogaster redii	KF974826.1
Amynthas amis	JX290429.1
Metaphire glareosa	AY960803.1
Metaphire tosaensis	AB542670.1
Aulodrilus pluriseta	LN810415.1
Tubifex montanus	LN810298.1
Mytilus_edulis	KU906108.1
Hirudo_medicinalis	HQ333519.1

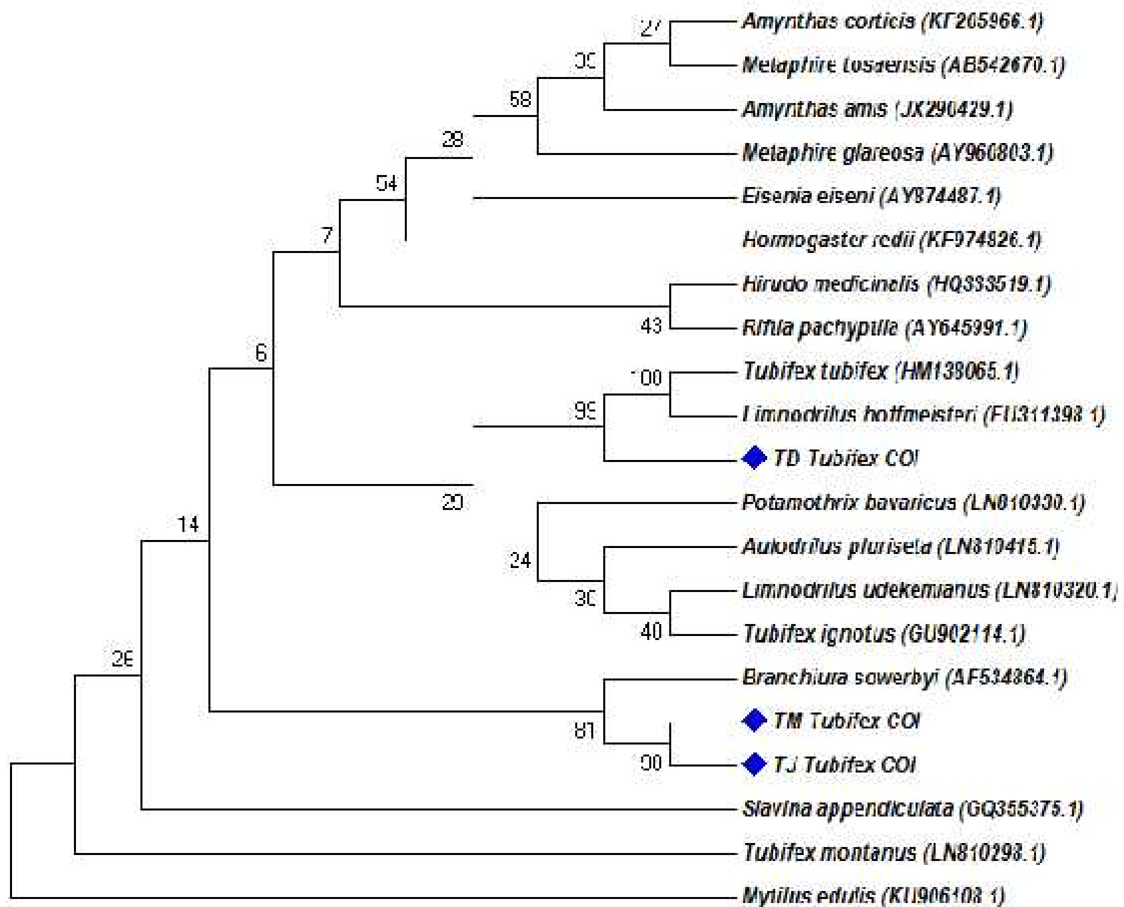


Figure 13 The neighbor-joining Phylogenetic tree were based on partial COI gene sequences. The evolutionary distances were compared by Maximum Composite Likelihood method. Number in tree are bootstrap values.

Phylogeny test

Test of phylogeny: Bootstrap method

No. of Bootstrap Replication: 1000

Substitutions type: Nucleotide

Model/ Method : Maximum Composite Likelihood

Substitutions to include d: Transitions + Transversions

Rates among sites: Uniform rates

Pattern among lineages: Same (Homogenous)

3.5.216S rDNA sequence based identification

3.5.2.1 PCR amplification

The extracted DNA from tubificid worms of three different region (Dhaka denoted by TD, Mymensing indicated by TM, Jessore denoted by TJ) was amplified by PCR amplification for 16S rDNA gene using 16S rDNA specific primers 16sar and 16sbr. The representative gel autoradiograph of the amplicon is showed in **Figure 14.**

3.5.2.2 Sequence output

From the chromatogram, the sequence data were transferred to fasta format and blasted within nucleotide database to identify species. 3 samples were sequenced and blasted. The 16S rDNA region of all samples was successfully amplified using PCR. The comprehensive identification results were based on Genbank databases. Database revealed that identity matches in the range of 93-99% for consensus sequences of 3 sample tubifex species. Genbank based identification showed that species alignment E value is 4e-173 for TD_Tubifex_16SrDNA, 1e-143 TJ_Tubifex_16SrDNA and 0.0 for TM_tubifex_16SrDNA.

Table 18 16S rDNA sequence based identification of representative tubificid worms from three different region

Sample ID	Identified species	Max score	Total score	Query cover (%)	E value	Identity (%)	Accession
TD_Tubifex_16SrDNA	*Limnodrilus hoffmeisteri*	617	617	100	4e-173	98	EU160485.1
TM_Tubifex_16SrDNA	*Limnodrilus hoffmeisteri*	645	645	100	0.0	99	EU117546.1
TJ_Tubifex_16SrDNA	*Limnodrilus hoffmeisteri*	520	520	98	1e-143	93	EU160485.1

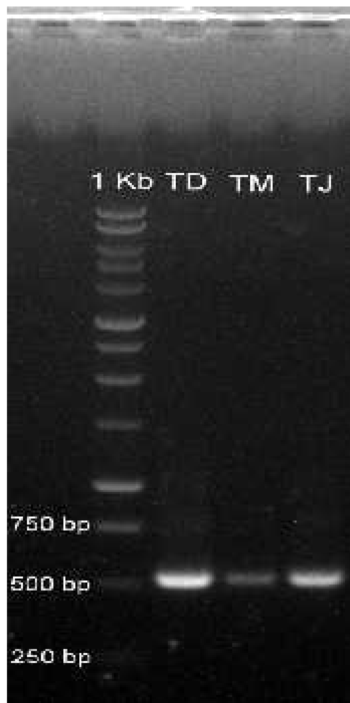
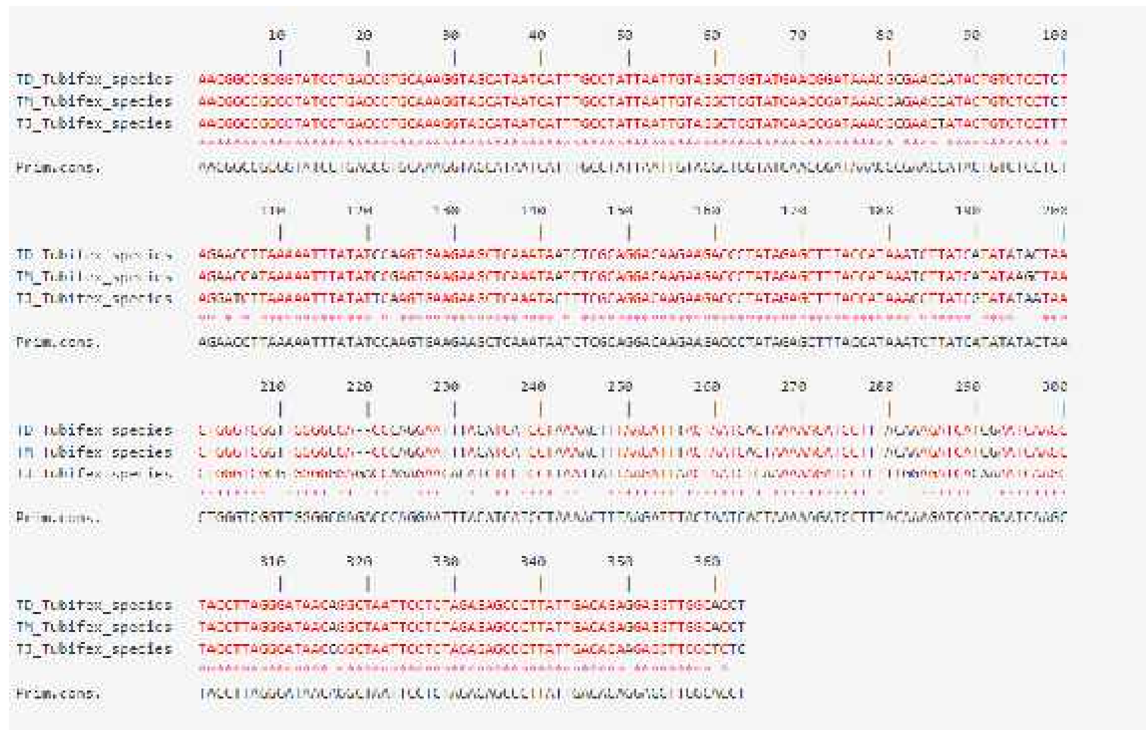


Figure 14 PCR amplification product profiles of 16S rDNA primer generated from 3 different Samples: TD,TM,TJ Here, 1 kb marker was used. Sample ID TD, TD_Tubifex_16SrDNA; TM, TM_Tubifex_16S rDNA; TJ, TJ_Tubifex_16SrDNA.

3.5.2.3 DNA sequence alignment

3.5.2.3.1 Alignment among Sample TD_Tubifex_16SrDNA, TM_Tubifex_16SrDNA and TJ_Tubifex_16SrDNA



Alignment length 363
Identity(*): 314 is 86.50%
Different: 49 is 13.50%

Figure 15 Multiple sequence alignment of 16S rDNA gene fragment of three samples TD_Tubifex_16SrDNA, TM_Tubifex_16SrDNA, TJ_Tubifex_16SrDNA where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 100)

Multiple sequence analysis is shown by view CLUSTALW to compare the sequences. After comparing the obtained sequence of 3 samples of tubificid worms, total 49 sites were found polymorphic. Therefore, the dissimilarity was 13.50%.

3.5.2.3.2 Alignment between TD_Tubifex_16SrDNA and TM_Tubifex_16SrDNA

Alignment Data

Alignment length 361

Identity(*): 356 is 98.61%

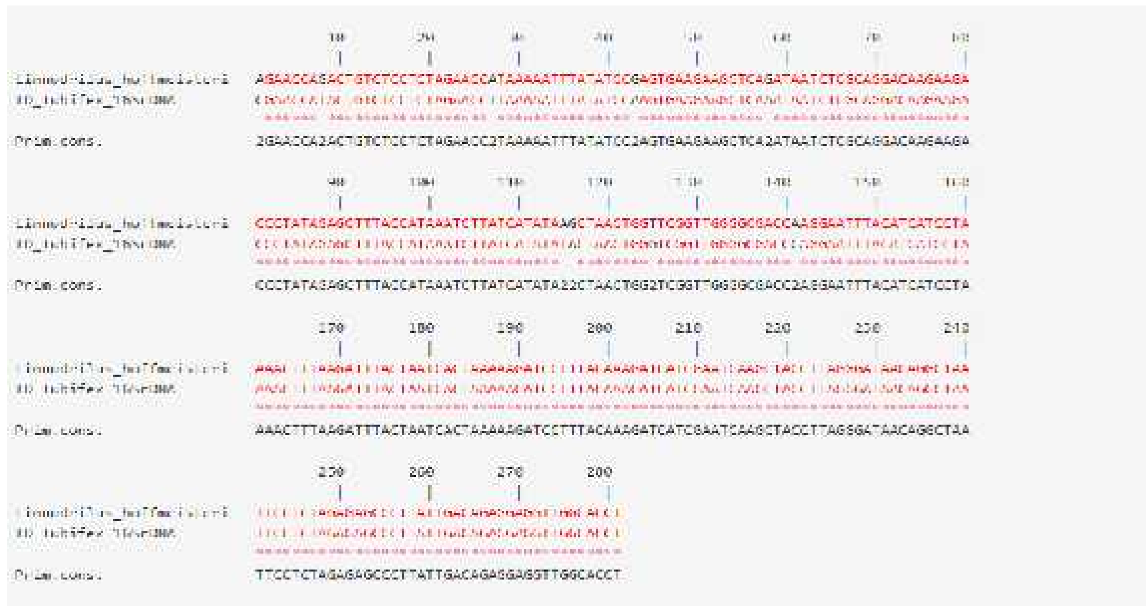
Different: 5 is 1.39%

Figure 16 Pairwise sequence alignment of 16S rDNA gene fragment of closely related species TD_Tubifex_16SrDNA and TM_Tubifex_16SrDNA where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 100)

Table 19 Polymorphic sites observed between closely related species TD_Tubifex_16SrDNA and TM_Tubifex_16SrDNA

Position	TD_Tubifex_16SrDNA	TM_Tubifex_16SrDNA
81	C	A
107	T	A
123	A	G
195	T	A
196	A	G

3.5.2.3.3 Alignment between TD_Tubifex_16SrDNA and *Limnodrilus hoffmeisteri*



Alignment Data

Alignment length 281

Identity(*): 272 is 96.80%

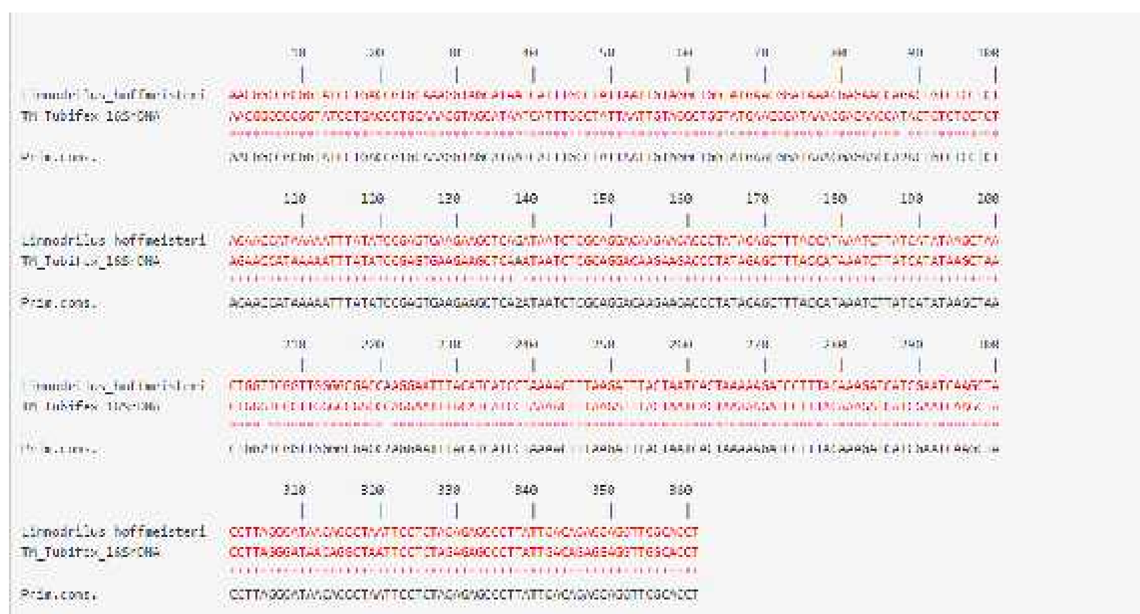
Different: 9 is 3.20%

Figure 17 Pairwise sequence alignment of 16SrDNA gene fragment of TD_Tubifex_16SrDNAand GenBank reference *Limnodrilus hoffmeisteri* where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 80)

Table 20 Polymorphic sites observed between TD_Tubifex_16SrDNA and *Limnodrilus hoffmeisteri*

Position	TD_Tubifex_16SrDNA	*Limnodrilus hoffmeisteri*
1	A	C
8	G	T
27	A	T
43	G	A
58	G	A
115	A	T
116	G	A
141	T	G

3.5.2.3.4 Alignment between TM_Tubifex_16SrDNA and *Limnodrilus hoffmeisteri*



Alignment Data

Alignment length 361

Identity(*): 357 is 98.89%

Different: 4 is 1.11%

Figure 18 Pairwise sequence alignment of 16SrDNA gene fragment of TM_Tubifex_16SrDNAand GenBank reference *Limnodrilus hoffmeisteri* where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 100)

Table 21 Polymorphic sites observed between TM_Tubifex_16SrDNA and *Limnodrilus hoffmeisteri*

Position	TM_Tubifex_16SrDNA	*Limnodrilus hoffmeisteri*
88	G	T
138	G	A
205	T	G
221	A	C

3.5.2.3.5 Alignment between TJ_Tubifex_16SrDNA and *Limnodrilus hoffmeisteri*

Alignment Data

Alignment length 365

Identity(*): 334 is 91.51%

Different: 31 is 8.49%

Figure 19 Pairwise sequence alignment of 16SrDNA gene fragment of TJ_Tubifex_16SrDNAand GenBank reference *Limnodrilus hoffmeisteri* where black among the red color indicates polymorphic positions. (CLUSTALW, alignment width 100)

Table 22 Polymorphic sites observed between TJ_Tubifex_16SrDNAand *Limnodrilus hoffmeisteri*

Position	TJ_Tubifex_16SrDNA	*Limnodrilus hoffmeisteri*
63	G	A
71	C	T
75	T	C
106	C	A
192	T	A
239	C	A
294	A	G
318	G	A
352	A	G

3.5.2.4 Phylogentic analysis

Multiple sequence analysis was done using examined sequences and downloaded (Table 23) sequences and phylogenetic tree was constructed. Phylogenetic analysis based on 16S rDNA gene sequences of the extracted DNA from 3 sample tubificid worms using neighbor joining confirmed the taxonomic position of the worms. From the tree it is clearly showed that TM_Tubifex_COI and TD_Tubifex_COI is closely related with *Limnodrilus hoffmeisteri* and TJ_Tubifex_COI is closely related with *Tubifex ignotus* and *Ilyodrilus templetoni.*

Table 23 List of all downloaded species from NCBI GenBank to support the phylogenetic analysis

Downloaded species	GenBank Accession no
Limnodrilus hoffmeisteri	EU160485.1
Limnodrilus cervix	AF325983.
Limnodrilus udekemianus	AY885612.1
Tubifex tubifex	HQ603822.1
Tubifex ignotus	AF325988.1
Ilyodrilus templetoni	EF089341.1
Psammoryctides barbatus	HM459993.1
Varichaetadrilus bizkaiensis	HQ603821.1
Metaphire glareosa	AY960816.1
Hormogaster redii	KF975176.1
Hirudo medicinalis	AF315058.1
Riftia pachyptila	AF315050.1
Potamothrix bavaricus	EU117509.1
Aulodrilus pluriseta	HM459991.1
Branchiura_sowerbyi	DQ459957.1
Slavina appendiculata	GQ355418.1
Mytilus edulis	U22868.1

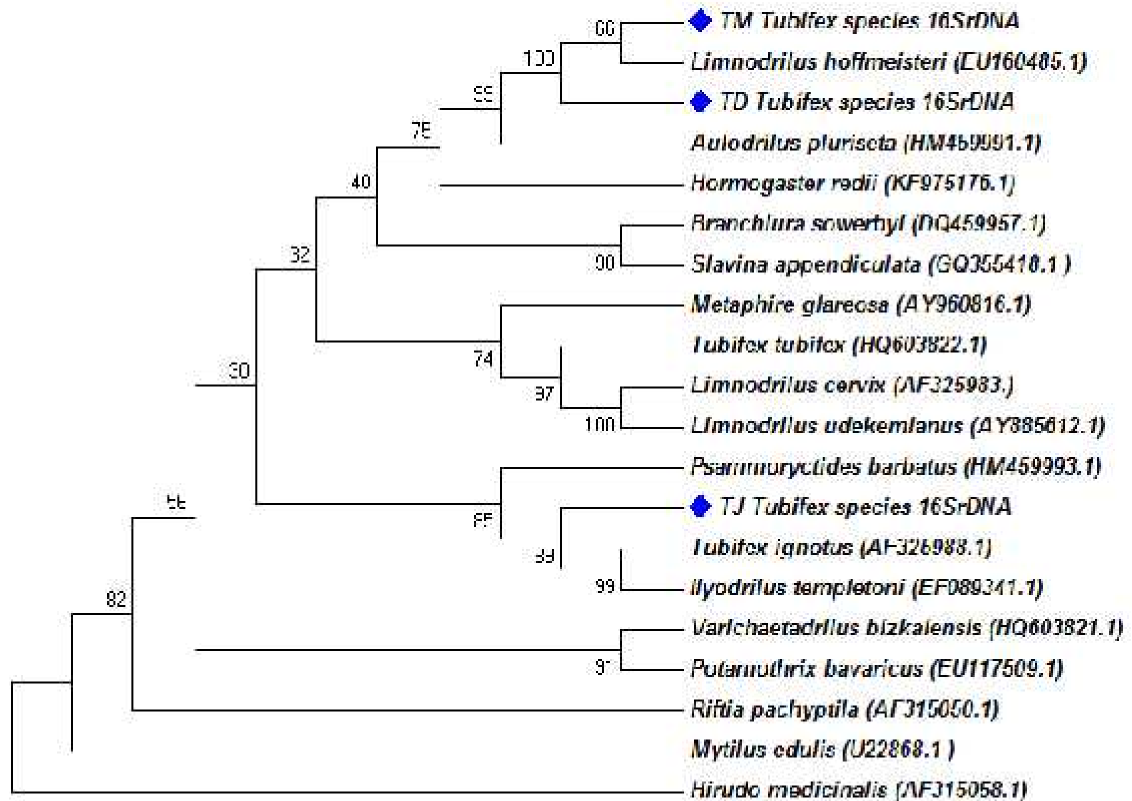


Figure 20 The neighbor-joining Phylogenetic tree was based on partial 16S rDNA gene sequences. The evolutionary distances were compared by Maximum Composite Likelihood method. Numbers in tree are bootstrap values.

Phylogeny test

Test of phylogeny: Bootstrap method

No. of Bootstrap Replication: 1000

Substitution type: Nucleotide

Model/ Method: Maximum Composite Likelihood

Substitutions to include d: Transitions + Transversions

Rates among sites: Uniform rates

Pattern among lineages: Same (Homogenous)

Chapter 4
Discussion

The observed highest yield of worms raised in the media ingredients wetted in blood could have resulted due to highest level of protein (58%) given in the feed stuffs over a culture duration of only 42 days. High level of inoculums at 50 mg cm^{-2}might have grown and propagated quickly with high protein level that could have played key role in getting maturity and reproduction to increase the biomass. Islam, Rahman, Mariom, Mollah and Siddik (2015) found only 256 mg cm^{-2}after 40 days culture period when chicken blood has been used as the wet medium in which inoculums was only 1.25 mg cm^{-2}. Moreover, they cultured the worms for a period of 70 days which is 1.75-times higher than the duration in this study that is associated with cost related to electricity to pump water. In addition, they renewed the system with media ingredients at 250 mg cm^{-2} while we use only 50 mg cm^{-2} for the first 2 weeks and 100 mg cm^{-2} in the next four weeks. Furthermore, Islam *etal*. (2015) used 20% MOC, 20% wheat bran (WB), 30% SBM, 20% cow dung (CD) and 10% sand while we used 30% SBM, 40% MOC and 30% mud. WB, CD and sand have been discarded in the present study because WB did not decomposed over 7-day period of wetting, CD has been found to enhance fungal growth and worms have been found to distribute evenly when mud was applied 1 day later the application of media ingredients. Evenly distribution of worms over the media ensures better utilization of the media. In our study, the media requirements reduced by 1.33-times than those of Mariom and Mollah (2012), Hossain *etal*. (2012) and Islam *etal*. (2015). Worms (616 mg cm^{-2} over 42-day culture duration) rose in the media wetted in rice gruel is also higher with the findings of Hossain *etal*. (2012) 210 mg cm^{-2}, Mariom & Mollah (2013) 430 mg cm^{-2} and Islam et al. (2015) 256 mg cm^{-2} over 40-day culture duration by inoculating 5, 1.25 and 1.25 mg cm^{-2}, respectively.

The highest level of whole body protein in the worms raised in the media ingredients wetted in cattle blood could be due to the maximum protein content of the media ingredients.

All 8 EAAs have been detected in the worms raised in the media ingredients wetted in blood that are suitable for raising fish requiring high level of protein (55%) in the diet (Tacon 1987). Fish need 2.37% arginine, 1.00% histidine, 1.54% isoleucine, 2.81% leucine, 3.25% lysine, 1.06% methonine, 1.77% threonine and 1.83% valine if the diet contains protein as high as 55%. The observed high level of all 14 amino acids in the worms could have resulted because of high level of amino acids found in the diets. Khan and Abidi (2014) have demonstrated that stinging catfish fry needs 1.6% dietary histidine for maximum growth which denotes the suitability of tubificid worms as larval food. Juvenilegiant croaker *Nibea japonica* had highest growth rate when fed feed with arginine 5.13%, histidine 2.91%, isoleucine 4.36%, leucine 8.94%, lysine 8.08%, methionine 2.86%, phenylalanine 4.58%, threonine 4.30%, tryptophan 1.28% and valine 5.51% (Cheng et al. 2016).

Table 24Inoculum size, cost of productionand harvest over 40 day culture duration in comparison to present one.

Inoculums (mg cm^{-2})	Media required to produce 1 kg worms		Production in 40 days of culture duration		References
	Media (kg)	Cost (BDT)	Wet medium	Standing biomass mg cm^{-2}	
1.25	0.80	19.2	Rice gruel	430	Mariom & Mollah, 2013
1.25	0.71	18	Chicken blood	256	Islam et al., 2015
			Rice gruel	243	
1.25	1.99	29.85	Water	280	Mollah et al., 2012
5	2.43	37	Water	106	Hossain et al., 2012
			Rice gruel	210	
			Water: Rice gruel (1:1)	169	
50	0.77	27.46	Cattle blood	683	Present study
	0.85	30.32	Rice gruel	615	
	0.90	32.10	Water	584	

Molecular identification of tubificid worms

COI sequenced based identification

Multiple Sequence Alignment has been performed for the 3 samples of tubifecid worms after comparing 3 sample TD_Tubifex_COI, TM_Tubifex_COI and TJ_Tubifex_COI it is found that total 154 sites were polymorphic.Therefore, the dissimilarity was 24.22%. Among 3 sequences TJ_Tubifex_COI (cultured) was closely related to TM_Tubifex_COI while less relationship was found with TJ_Tubifex_COI and TD_Tubifex_COI.

After comparingCOI gene sequence of two closely related species of Tubificid worms TM_Tubifex_COI and TJ_Tubifex_COI it was found that 62 out of 630 bp nucleotide bases of the sequence were polymorphic.

COI gene sequence of sample TD_Tubifex_COI is compared with *Tubifex tubifex* (accession no. HM138065.1) from Gene Bank database. After comparing these sequences it was found that 83 out of 630 bp nucleotide bases of the sequence were polymorphic.

COI gene sequence of sample TM_Tubifex_COI is compared with *Tubifex tubifex* (accession no. EU311345.1) from Gene Bank database. After comparing these sequences it is found that 120 out of 630 bp nucleotide bases of the sequence were polymorphic.

COI gene sequence of sample TJ_Tubifex_COI is compared with *Branchiura sowerbyi* (accession no.AF534864.1) from Gene Bank database. After comparing these sequences it was found that 79 out of 630 bp nucleotide bases of the sequence were polymorphic.

Construction of phylogenetic tree involved a total of 21 (3 sample sequences with 18 downloaded sequences from NCBI GenBank) nucleotide sequences that support the findings of multiple sequence alignment. The percentage of replicates trees in which the associated taxa was clustered together with the bootstrap test where 1000 replication was used. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura et al. 1993). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. There are total 630 position in the final dataset. Evolutionary analysis is conducted in MEGA following the instruction of Tamura et al.(2013).

From molecular identification, it was found that sample TD_Tubifex_COI was 87% similar with *Tubifex tubifex*, TM_Tubifex_COI was 81% similar to *Tubifex tubifex*and TJ_Tubifex_COI (cultured) was 87% similar to *Branchiura sowerbyi.*

From the neighbor-joining phylogenetic tree, taxonomic position of the worms were confirmed. Phylogenetic tree have clearly demonstrated that TM_Tubifex_COI and TJ_Tubifex_COI was closely related with *Branchiura sowerbyi* and TD_Tubifex_COI was closely related with *Tubifex tubifex* and *Limnodrilus hoffmeisteri*.

16S rDNA sequenced based identification

Multiple Sequence Alignment has been performed for the 3 samples of tubifecid worms. After comparing 3 samples TD_Tubifex_16SrDNA, TM_Tubifex_16SrDNA and TJ_Tubifex_16SrDNA, it was found that total 49 sites were polymorphic.Therefore the dissimilarity was 13.50%. Among 3 sequences TD_Tubifex_16SrDNA (cultured) was closely related with TM_Tubifex_16SrDNA while less relation was found with TJ_Tubifex_16SrDNA (cultured).

After comparing 16S rDNA gene sequence of two closely related species of Tubificid worms TD_Tubifex_16SrDNA and TM_Tubifex_16SrDNA it has been found that 5 (81,107,123,195,196) sites out of 363 bp nucleotide bases of the sequence are polymorphic.

16S rDNA gene sequence of sample TD_Tubifex_16SrDNA was compared with *Limnodrilus hoffmeisteri* (accession no.EU160485,1) from GenBank database. After comparing these sequences it has been found that 9 (1,8,27,43,58,115,116,125,141) out of 281 bp nucleotide bases of the sequence are polymorphic.

16S rDNA gene sequence of sample TM_Tubifex_16SrDNA was compared with*Limnodrilus hoffmeisteri*(accession no. EU160485,1) from GenBank database. After comparing these sequences it has been found that 120 out of 630 bp nucleotide bases of the sequence are polymorphic.

16S rDNA gene sequence of sample TJ_Tubifex_16SrDNAwas compared with *Limnodrilus hoffmeisteri* (accession no EU117546.1) from Gene Bank database. After comparing these sequences it has been found that 79 out of 630 bp nucleotide bases of the sequence were polymorphic.

Construction of phylogenetic tree involved a total of 21 (3 sample sequences with 17 downloaded sequences from NCBI GenBank) nucleotide sequences that upports the findings of multiple sequence alignment. The percentage of replicates trees in which the associated taxa was clustered together with the bootstrap test where 1000 replication were used. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura et al. 1993). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. There are total 363 positions in the final dataset. Evolutionary analysis is conducted in MEGA following the instruction of (Tamura et al. 2013).

From molecular identification, it has been found that sample TD_Tubifex_16SrDNA was 98% similar with *Limnodrilus hoffmeisteri*, TM_Tubifex_16SrDNA was 99% similar to *Limnodrilus hoffmeisteri* and TJ_Tubifex_16SrDNA (cultured) was 93% similar to *Limnodrilus hoffmeisteri.*

From the neighbor-joining phylogenetic tree, taxonomic positions of the worms were confirmed. Phylogenetic tree have clearly demonstrated that TM_Tubifex_16SrDNA and TD_Tubifex_16SrDNA were closely related with *Limnodrilus hoffmeisteri* and TJ_Tubifex_16SrDNA was closely related with *Tubifex ignotus* and *Ilyodrilus templetoni.*

Chapter 5
Conclusions and Recommendations

Chapter 5
Conclusionsand Recommendations

5.1 Conclusions

This study has clearly demonstrated that tubificid worms can be cultured commercially at farmer level by using the mixture of 40% mustard oil cake, 30% wheat bran and 30% mud over a duration of 42 days. Cattle blood can be used as wet medium to increase yield. Culture system renewal is 50 mg cm^{-2} by media ingredients once for the first 2 weeks and twice for the rest 4 weeks. Tubificid worms contain all essential amino acids at high level required for high protein needed fishes.

COI gene sequencing revealed that sample TD_Tubifex_COI was 87% similar to *Tubifex tubifex*, TM_Tubifex_COI was 81% similar to *Tubifex tubifex* and TJ_Tubifex_COI (cultured) was 87% similar to *Branchiura sowerbyi.* However, 16S rDNA sequencing showed that sample TD_Tubifex_16SrDNA was 98% similar to *Limnodrilus hoffmeisteri*, TM_Tubifex_16SrDNA was 99% similar to *Limnodrilus hoffmeisteri* and TJ_Tubifex_16SrDNA (cultured) was 93% similar to *Limnodrilus hoffmeisteri.*

5.2 Recommendations

Since this is the first attempt to identify to memorialize the culture of tubificid worms at farmer level, before disseminating the technique to the farmers, culture technique need to be confirmed through piloting the technique by repeating over several seasons mostly between March and October.

Mixed samples of tubificid worms were drawn and used this present study from Dhaka, Jessore and Mymensingh districts because of incapability in identifying the worms morphometrically. Therefore, Cloning technique by using cloning kit (TOPO10, Invitrogen) can be used for individual sample analysis by using COI and 16S rDNA genes.

References

References

Ahmed, M.T., Mollah, M.F.A., 1992. Effects of various levels of wheat bran and mustard oil cake in the culture media on tubificid production. Aquaculture 107, 107-113.

Aston, R.J., Milner A.G.P., 1981. Conditions required for the culture of Branchiura sowerbyi (Oligochaeta: Tubificidae) in activated sludge. Aquaculture26, 155-160.

AOAC, 2000. Official methods of analysis of AOAC.International 17th edition; Gaithersburg, MD, USA Association of Analytical Communities.

Barnes, R.D., 1966. Invertebrate Zoology. W.B. Saunders Company, Philadelphia,London. 362.

Beard, C.B., Hamm, D.M., Collins, F.H., 1993. The mitochondrial genome of the mosquito *Anophelesgambiae*: DNA sequence, genome organization and comparisons with mitochondrial sequences of other insects. Insect Mol. Biol.2, 103-124.

Beauchamp, Katherine, A., Terry, S., McDowell, R.P.H., 2001. Molecular phylogeny of tubificid oligochaetes with special emphasis on Tubifex tubifex (Tubificidae). Mol. Phylogenet. Evol. 19, 216-224.

Begum, M., Ahmed, K.N., Sultana, M.R., Hasan, M., 2015. Development of a culture techniques for Tubificid worm, under laboratory conditions. Bangladesh J. Zool. 42, 117-122.

Bely, Alexandra, E., Gregory, A., Wray, 2004. Molecular phylogeny of naidid worms (Annelida: Clitellata) based on cytochrome oxidase I. Mol. Phylogenet. Evol. 30, 50-63.

Brinkhurst, R.O., 1971. A guide for the identification of British aquatic Oligochaeta. Kendal, Wilson: Freshwater Biological Association.

Brinkhurst, R.O., Kennedy, C.R., 1965. Studies on the biology of Tubificidae (Annelida, Oligochaeta) in a polluted stream. J. Anim. Ecol. 34, 429-443.

Cheng, C.R., Wan, J.T., Han, T., Li, X.Y, Jiang, Y.D., Wen, X.B., 2016. Effects of different dietary amino acid patterns on growth performance and body composition of juvenile giant croaker Nibea japonica. Aquacult. Res. 47, 3942 – 3951.

Cromwell, D.G., 2012. Soybean Meal–An Exceptional Protein Source. Soybean Meal Info Center.

Cromwell, G.L., Calvert, C.C., Cline, T.R., Crenshaw, J.D., Crenshaw, T.D., Easter, R.A., Ewan, R.C., Hamilton, C.R., Hill, G.M., Lewis, A.J., Mahan, D.C., Miller,

E.R.J.L., Nelssen, J.E., Pettigrew, L.F., Tribble, T.L., Veum, Yen, J.T., NCR-42 Committee on Swine Nutrition., 1999. Variability among sources and laboratories in nutrient analyses of corn and soybean meal. J. Anim. Sci. 77, 3262-3273.

Crottini, A., Marotta, R., Barbuto, M., Casiraghi, M., Ferrauti, M., 2008. The world in a river? A preliminary analysis of the 16S rDNA variability of Tubifex species (Clitellata: Tubificidae) from the Lambro River. Mol. Phylogenet. Evol.48, 1189-1203.

Derycke, S., Vanaverbeke, j., Rigaux, A., Backeljau, T., Mones, T., 2010. Exploring the use of cytochrome oxidase c subunit 1 (COI) for DNA barcoding of free-living marine nematodes. PLoS One 5, 13716.

Fisher, J.B., Matisoff, G., 1981. High resolution vertical profiles of pH in recent sediments. Hydrobiologia. 79, 277–284.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R.,1994. DNA primers for amplification ofmitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3, 294-299.

FRSS, 2016. Fisheries Statistical Yearbook of Bangladesh. Fisheries Resources Survey System (FRSS), Department of Fisheries, Bangladesh. 32, 57.

Hallett, S.L., Stephen, D.A., Erseus, C., El-Matbouli, M., 2005, Dissemination of triactinomyxons (Myxozoa) via oligochaetes used as live food for aquarium fishes. Dis. Aquat. Org. 65.2, 137-152.

Hasan, M., 2003. Growth and feed utilization of tilapia (*Oreochromisniloticus* Linn.) fed with different protein sources with and without vitamin. The Dhaka Uni. J. Biol. Sci.12, 105-113.

Hebert, P.D.N., Cywinska, A., Ball, S.L., Dewaard, J.R., J.R. 2003*a*. Biological identifications through DNA barcodes.Proc. Royal Soc. London B: Biol. Sci. 270. 96-S99.

Hebert, P.D.N., Ratnasingham, S., Dewaard, J.R., 2003B. Barcoding animal life: Cytochrome Coxidase subunit 1 divergences among closely related species. Proc. Royal Soc. London B: Biol. Sci. 270, 1-4.

Hebert, P.D.N., Ratnasingham, S., Jeremy, R.W., 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc. Royal Soc. London B: Biol. Sci. 270,96-S99.

Hossain, A., Mollah, M.F.A., Hasan M., 2012. Ratio optimization of media ingredients for mass culture of Tubificid worms (Oligochaeta, Tubificidae) in Bangladesh. Asian Fish. Sci. 25, 357-368.

Hossain, A., Rahman, M,S., Hasan M., 2011. Optimum harvest for sustainable yield of fish live food tubificid worms. The Dhaka Univ. J. Biol. Sci. 20.1, 57-63.

Howell, N., 1989. Evolutionary conservation of protein regions in the proton motive cytochrome b and their possible roles in redox catalysis. J. Mol. Evol. 29, 157-69.

http://www.soymeal.org/ReviewPapers/SBMExceptionalProteinSource.pdf

Huq, S.M.I., Alam, M.D., 2005(b). A handbook on analyses of soil, plant and water. Bangladesh-Australia centre for environmental research. University of Dhaka,Bangladesh. 246.

Islam, S., Rahman, M.M., Mariom, Mollah F.A., Siddik, M.A.B., 2015. Performance of chicken blood for the production of Tubificid worms as live food for fish. World Appl. Sci. J. 33, 496-502.

Janda, J.M., Sharon, L.A., 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. J. Clin. Microbiol. 45, 2761-2764.

Kaster, J.L., 1980. The productive biology of *Tubifex tibifex*Mullar (Annelida: Tubificidae). American Midland Naturalist 104, 364-366.

Khan, M.A, Abidi, S.F., 2014. Dietary histidine requirement of Singhi,Heteropneustes fossilis fry (Bloch).Aquacult. Res. 45, 1341–1354.

Kumar, S., Tamura,K., Nei,M., 1994. MEGA: Molecular Evolutionary Genetics Analysis software for microcomputers. Comput. Appl. Biosci. 10, 189–191.

Krodkiewska, Mariola, 2007. The distribution of Potamothrix bavaricus (Oeschmann, 1913) (Oligochaeta) in anthropogenic freshwater habitats of an industrialised area (Upper Silesia, Poland). Limnologica-Ecol. Man. Inland Waters 37, 259-263.

Mackie, G.L., 2001. Studies on the growth and reproduction of *Tubifex Tubifex* Muller. Acta Hydrobiologia Sinica. 25, 14-20.

Mariom, Mollah, M.F.A., 2013. Effects of culture space on the production of Tubificid worms (Oligochaeta, Naididae). Asian Fish. Sci. 26, 222-231.

Mariom, Mollah, M.F.A., 2012. Development of a Suitable Culture Medium for the Production of TubificidWorms. Asian Fish. Sci. 25,40-51.

Massol-Deya, Arturo, A., David A., Odelson, Robert, F., Hickey, James, M., Tiedje, 1995. Bacterial community fingerprinting of amplified 16S and 16–23S ribosomal DNA gene sequences and restriction endonuclease analysis ARDRA.Molecular microbial ecology manual. Springe Netherlands. 89-296.

Mollah, Ahamed, 1989. A note on Preliminary study of culture of Tubificid worms. Bngladesh J. Fish. 12, 91-95.

Mollah, M.F.A., Mosharaf, K., Mariom, 2012. Selection of suitable media and intervals of media inoculation for culturing Tubificid worms. J. Bangladesh Agric. Univ. 10, 325-330.

Park, S.H., Zhang, Y., Piao, H., Yu, D.H., Jeong, H.J., Yoo, G.Y., Chung, U., Jo, T., Hwang, J., 2009. Use of cytochrome c oxidase subunit I (COI) nucleotide sequences for identification of the Korean Luciliinae fly species (Diptera: Calliphoridae) in forensic investigations.J.Korean MedSci. 24, 1058-1063.

Purkhold, U., Roser, P.A., Schmid, C.M., Koops, H.P., Wangnerl, M., 2000. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. Applied and Environ. Microbiol. 66, 368-5382.

Ramachandran, S., Singh, S.k., Larroche, C., Soccol, C.R., Pandey, A., 2007. Oil cakes and their biotechnological applications–A review. Biores. Ttechnol. 98, 10. 2000–2009.

Rech, K.C., Guereschi, R.M., Nuner, A.P.O., Sticca, C.S., 2013. Density and Organic Matter Influence on Egg-laying and Hatching of Branchiura sowerbyi (Oligochaeta). J. World Aquaculture Soc**.** 44, 267-272.

Rodriguez, P., Madrid, M.M., Arrate, J.A., Navarro, E., 2001. Selective feeding by the aquatic oligochaete Tubifex tubifex (Tubificidae, Clitellata). Hydrobiol. 463, 133-140.

Rodriguez, P., Trefor B.R., 2011. The pollution biology of aquatic oligochaetes. Springer Science & Business Media. 87-158.

Sarker, A.K., Saha, D., Begum, H., Zaman, A., Rahman, M.M., 2015 Comparison of cake compositions, pepsin digestibility and amino acids concentration of proteins isolated from black mustard and yellow mustard cakes**.** AMB Express 5:22.DOI 10.1186/s13568-015-0110-y.

Sjölin, E., Erséus, C., Källersjö, M., 2005. Phylogeny of Tubificidae (Annelida, Clitellata) based on mitochondrial and nuclear sequence data. Mol. Phylogenet. Evol. 35, 431-441.

Strüder, K., Michaela C., Denis, H.L., 2010. Comparative analysis of the mitochondrial cytochrome c oxidase subunit I (COI) gene in ciliates (Alveolata, Ciliophora) and evaluation of its suitability as a biodiversity marker. Systematics and Biodiversity. 8,131-148.

Stackebrandt, E., Goebel, B.M., 1994. Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol.44, 846–9.

Tacon, G.J.A., 1987. The nutrition and feeding of farmed fish and shrimp – A training manual 1. The essential nutrients. FAO**.**22-23.

Tamura K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA) 101, 11030-11035.

Tamura, K., Stecher, G., Peterson, D., Filipski A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution.30, 2725-2729.

Timm, T., 2013. The genus Potamothrix (Annelida, Oligochaeta, Tubificidae): a literature review. Estonian J. Ecol. 62, 121-136.

Timm, T., 2012Life forms in Oligochaeta: a literature review. Zoology in the Middle East 58, 71-82.

Tobe, S.S., Kitchener A,C., Linacre A.M., 2010. Reconstructing mammalian phylogenies: a detailed comparison of the cytochrome b and cytochrome oxidase subunit I mitochondrial genes. PloS one 5, 14156.

Valnot, I., Osmond, S., Gigarel, N., Mehaye, B., Amiel, J., Cormier-Daire, V., Munnich, A., Bonnefont, J. P., Rustin, P.,Rötig. A., 2000. Mutations of the SCO1 Gene in Mitochondrial Cytochrome *c* Oxidase Deficiency with Neonatal Onset Hepatic Failure and Encephalopathy. Am. J. Hum. Genet. 67, 1104-1109

Vivien, R., Wyler, S., Lafont, M., Pawlowski, J., 2015. Molecular barcoding of aquatic oligochaetes: implications for biomonitoring. PloS one. 10, 125485.

Woo, P.C.Y., Leung, P.K.L., Leung, K.W., Yuen, K.Y., 2000. Identification by 16s

ribosomal RNA gene sequencing of an Enterobacteriaceae species from a bone marrow transplant recipient,J. Clin. Pathol. 53.4,211–215.

Yokoyama, H., Yasoja S., Liyanage, Hisatsugu, W., 2002. A new strategy against myxosporean diseases: vector control in hemorrhagic thelohanellosis of cultured carp.Fish. Sci. 68, 825-828.

Appendices

Appendices

Appendix A

Mean ± SEM of the yield of tubificid worms raised in the media ingredients wetted in cattle blood, rice gruel and subsurface clean water.

				mg_cm2
Wet medium	Blood used as wet medium	1		679.86
		2		680.21
		3		690.97
		Total	Mean	683.68
			SEM	3.65
	Rice gruel used as wet medium	1		611.46
		2		612.50
		3		622.92
		Total	Mean	615.63
			SEM	3.66
	Water used as wet medium	1		586.81
		2		584.38
		3		581.94
		Total	Mean	584.38
			SEM	1.41
	Total	Mean		627.89
		SEM		14.74

Appendix B

Anova table to compare means of tubificid worms' yield

ANOVA

mg_cm2

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	15468.971	2	7734.485	269.861	.000
Within Groups	171.966	6	28.661		
Total	15640.936	8			

Appendix C
Post Hoc Tests for multiple comparison

Homogeneous Subsets

mg_cm2

	Wet medium	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	Water used as wet medium	3	584.3767		
	Rice gruel used as wet medium	3		615.6267	
	Blood used as wet medium	3			683.6800
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Appendix D

Mean ± SEM of the amino acids of the wild and cultured worms rose on the media ingredients wetted in cattle blood, rice gruel and water.

				Aspartic acid	Threonine	Serine	Glutamic acid	Isoleucine	Glycine	Alanine	Valine	Methionine	Leucine	Tyrosine	Histidine	Lysine	Arginine
worms	Wild	1		3.50	1.70	3.10	4.90	2.50	6.10	4.70	2.60	2.70	4.50	2.50	1.80	6.40	5.80
		2		4.01	1.68	3.98	4.89	2.47	6.10	4.68	2.57	2.67	4.46	2.52	1.76	6.31	5.76
		3		3.65	1.73	3.12	4.94	2.51	6.19	4.73	2.62	2.72	4.53	2.48	1.83	6.52	5.83
		Total	Mean	3.7200	1.7033	3.4000	2.4933	2.4933	6.1300	4.7033	2.5967	2.6967	4.4967	2.5000	1.7967	6.4100	5.7967
			SEM	.15133	.01453	.29006	.01202	.01202	.03000	.01453	.01453	.01453	.02028	.01155	.02028	.06083	.02028
	Blood	1		4.70	3.60	4.20	5.90	4.50	7.00	5.80	3.70	3.40	5.20	3.60	2.40	6.90	6.40
		2		4.91	3.67	4.18	5.88	4.47	6.70	5.78	3.73	3.38	5.15	3.63	2.43	6.77	6.37
		3		4.83	3.58	4.23	5.95	4.53	7.40	5.83	3.68	3.43	5.23	3.59	2.39	6.93	6.43
		Total	Mean	4.8133	3.6167	4.2033	4.5000	4.5000	7.0333	5.8033	3.7033	3.4033	5.1933	3.6067	2.4067	6.8667	6.4000
			SEM	.06119	.02728	.01453	.01732	.01732	.20276	.01453	.01453	.01453	.02333	.01202	.01202	.04910	.01732
	Gruel	1		3.60	2.70	3.20	4.80	2.80	5.40	4.80	2.20	2.50	4.20	2.40	1.60	5.80	5.40
		2		3.46	2.74	3.21	4.77	2.77	5.39	4.77	2.18	2.51	4.18	2.43	1.55	5.77	5.32
		3		3.62	2.69	3.22	4.82	2.82	5.42	4.83	2.23	2.47	4.23	2.37	1.64	5.83	5.43
		Total	Mean	3.5600	2.7100	3.2100	2.7967	2.7967	5.4033	4.8000	2.2033	2.4933	4.2033	2.4000	1.5967	5.8000	5.3833
			SEM	.05033	.01528	.00577	.01453	.01453	.00882	.01732	.01453	.01202	.01453	.01732	.02603	.01732	.03283
	Water	1		3.40	2.60	3.00	4.50	2.50	5.30	4.60	2.20	2.30	3.80	2.10	1.50	5.60	4.80
		2		3.34	2.64	2.98	4.47	2.53	5.32	4.57	2.19	2.27	3.78	2.90	1.47	5.63	4.69
		3		3.42	2.58	3.10	4.52	2.49	5.27	4.63	2.22	2.33	3.82	2.12	1.52	5.57	4.83
		Total	Mean	3.3867	2.6067	3.0267	2.5067	2.5067	5.2967	4.6000	2.2033	2.3000	3.8000	2.3733	1.4967	5.6000	4.7733
			SEM	.02404	.01764	.03712	.01202	.01202	.01453	.01732	.00882	.01732	.01155	.26340	.01453	.01732	.04256
	Total	Mean		3.8700	2.6592	3.4600	5.0283	3.0742	5.9658	4.9767	2.6767	2.7233	4.4233	2.7200	1.8242	6.1692	5.5883
		SEM		.17199	.20443	.14908	.16025	.25095	.21401	.14564	.18525	.12586	.15358	.16494	.10681	.15212	.17937

Appendix E

ANOVA table to compare means of amino acids of the wild and cultured worms rose in the media ingredients wetted in blood, rice gruel and water.

Tests of Between-Subjects Effects

Source	Dependent Variable	Type I Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Asparticacid	3.726[a]	3	1.242	55.657	.000
	Threonine	5.507[b]	3	1.836	1631.790	.000
	Serine	2.419[c]	3	.806	12.538	.002
	Glutamicacid	3.383[d]	3	1.128	1380.830	.000
	Glycine	5.792[e]	3	1.931	60.857	.000
	Alanine	2.794[f]	3	.931	1214.580	.000
	Valine	4.526[g]	3	1.509	2828.500	.000
	Methionine	2.086[h]	3	.695	1069.573	.000
	Isoleucine	8.308[i]	3	2.769	4615.718	.000
	Leucine	3.106[j]	3	1.035	1061.766	.000
	Tyrosine	3.171[k]	3	1.057	20.149	.000
	Histidine	1.497[l]	3	.499	460.685	.000
	Lysine	3.014[m]	3	1.005	199.618	.000
	Arginine	4.225[n]	3	1.408	521.650	.000
Intercept	Asparticacid	179.723	1	179.723	8053.299	.000
	Threonine	84.854	1	84.854	75425.785	.000
	Serine	143.659	1	143.659	2233.623	.000
	Glutamicacid	303.410	1	303.410	371522.000	.000
	Glycine	427.094	1	427.094	13462.380	.000
	Alanine	297.207	1	297.207	387660.696	.000
	Valine	85.975	1	85.975	161202.250	.000
	Methionine	88.999	1	88.999	136920.821	.000
	Isoleucine	113.406	1	113.406	189010.014	.000
	Leucine	234.791	1	234.791	240810.803	.000
	Tyrosine	88.781	1	88.781	1692.137	.000
	Histidine	39.931	1	39.931	36859.392	.000
	Lysine	456.703	1	456.703	90735.776	.000
	Arginine	374.754	1	374.754	138797.642	.000
worms	Asparticacid	3.726	3	1.242	55.657	.000
	Threonine	5.507	3	1.836	1631.790	.000
	Serine	2.419	3	.806	12.538	.002
	Glutamicacid	3.383	3	1.128	1380.830	.000
	Glycine	5.792	3	1.931	60.857	.000
	Alanine	2.794	3	.931	1214.580	.000
	Valine	4.526	3	1.509	2828.500	.000

	Methionine	2.086	3	.695	1069.573	.000
	Isoleucine	8.308	3	2.769	4615.718	.000
	Leucine	3.106	3	1.035	1061.766	.000
	Tyrosine	3.171	3	1.057	20.149	.000
	Histidine	1.497	3	.499	460.685	.000
	Lysine	3.014	3	1.005	199.618	.000
	Arginine	4.225	3	1.408	521.650	.000
Error	Asparticacid	.179	8	.022		
	Threonine	.009	8	.001		
	Serine	.515	8	.064		
	Glutamicacid	.007	8	.001		
	Glycine	.254	8	.032		
	Alanine	.006	8	.001		
	Valine	.004	8	.001		
	Methionine	.005	8	.001		
	Isoleucine	.005	8	.001		
	Leucine	.008	8	.001		
	Tyrosine	.420	8	.052		
	Histidine	.009	8	.001		
	Lysine	.040	8	.005		
	Arginine	.022	8	.003		
Total	Asparticacid	183.628	12			
	Threonine	90.370	12			
	Serine	146.593	12			
	Glutamicacid	306.799	12			
	Glycine	433.140	12			
	Alanine	300.006	12			
	Valine	90.504	12			
	Methionine	91.089	12			
	Isoleucine	121.719	12			
	Leucine	237.904	12			
	Tyrosine	92.372	12			
	Histidine	41.437	12			
	Lysine	459.758	12			
	Arginine	379.001	12			
Corrected Total	Asparticacid	3.905	11			
	Threonine	5.516	11			
	Serine	2.934	11			
	Glutamicacid	3.390	11			
	Glycine	6.046	11			
	Alanine	2.800	11			

	Valine	4.530	11			
	Methionine	2.091	11			
	Isoleucine	8.313	11			
	Leucine	3.113	11			
	Tyrosine	3.591	11			
	Histidine	1.506	11			
	Lysine	3.054	11			
	Arginine	4.247	11			
a. R Squared = .954 (Adjusted R Squared = .937)						
b. R Squared = .998 (Adjusted R Squared = .998)						
c. R Squared = .825 (Adjusted R Squared = .759)						
d. R Squared = .998 (Adjusted R Squared = .997)						
e. R Squared = .958 (Adjusted R Squared = .942)						
f. R Squared = .998 (Adjusted R Squared = .997)						
g. R Squared = .999 (Adjusted R Squared = .999)						
h. R Squared = .998 (Adjusted R Squared = .997)						
i. R Squared = .999 (Adjusted R Squared = .999)						
j. R Squared = .997 (Adjusted R Squared = .997)						
k. R Squared = .883 (Adjusted R Squared = .839)						
l. R Squared = .994 (Adjusted R Squared = .992)						
m. R Squared = .987 (Adjusted R Squared = .982)						
n. R Squared = .995 (Adjusted R Squared = .993)						

Appendix F

Post Hoc test for multiple comparisons of the amino acids

Asparticacid

	worms	N	Subset	
			1	2
Tukey HSD[a,b,c]	water	3	3.3867	
	gruel	3	3.5600	
	wild	3	3.7200	
	blood	3		4.8133
	Sig.		.097	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .022.

a. Uses Harmonic Mean Sample Size = 3.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Threonine

	worms	N	Subset			
			1	2	3	4
Tukey HSD[a,b,c]	wild	3	1.7033			
	water	3		2.6067		
	gruel	3			2.7100	
	blood	3				3.6167
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .001.

a. Uses Harmonic Mean Sample Size = 3.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Serine

	worms	N	Subset	
			1	2
Tukey HSD[a,b,c]	water	3	3.0267	
	gruel	3	3.2100	
	wild	3	3.4000	
	blood	3		4.2033
	Sig.		.338	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .064.

a. Uses Harmonic Mean Sample Size = 3.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Glutamicacid

	worms	N	Subset			
			1	2	3	4
Tukey HSD[a,b,c]	water	3	4.4967			
	gruel	3		4.7967		
	wild	3			4.9100	
	blood	3				5.9100
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .001.
a. Uses Harmonic Mean Sample Size = 3.000.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = .05.

Glycine

	worms	N	Subset		
			1	2	3
Tukey HSD[a,b,c]	water	3	5.2967		
	gruel	3	5.4033		
	wild	3		6.1300	
	blood	3			7.0333
	Sig.		.881	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .032.
a. Uses Harmonic Mean Sample Size = 3.000.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = .05.

Alanine

	worms	N	Subset			
			1	2	3	4
Tukey HSD[a,b,c]	water	3	4.6000			
	wild	3		4.7033		
	gruel	3			4.8000	
	blood	3				5.8033
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .001.

a. Uses Harmonic Mean Sample Size = 3.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Valine

	worms	N	Subset		
			1	2	3
Tukey HSD[a,b,c]	gruel	3	2.2033		
	water	3	2.2033		
	wild	3		2.5967	
	blood	3			3.7033
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .001.

a. Uses Harmonic Mean Sample Size = 3.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Methionine

	worms	N	Subset			
			1	2	3	4
Tukey HSD[a,b,c]	water	3	2.3000			
	gruel	3		2.4933		
	wild	3			2.6967	
	blood	3				3.4033
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .001.
a. Uses Harmonic Mean Sample Size = 3.000.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = .05.

Isoleucine

	worms	N	Subset		
			1	2	3
Tukey HSD[a,b,c]	wild	3	2.4933		
	water	3	2.5067		
	gruel	3		2.7967	
	blood	3			4.5000
	Sig.		.907	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .001.
a. Uses Harmonic Mean Sample Size = 3.000.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = .05.

Leucine

	worms	N	Subset			
			1	2	3	4
Tukey HSD[a,b,c]	water	3	3.8000			
	gruel	3		4.2033		
	wild	3			4.4967	
	blood	3				5.1933
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .001.
a. Uses Harmonic Mean Sample Size = 3.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Tyrosine

	worms	N	Subset	
			1	2
Tukey HSD[a,b,c]	water	3	2.3733	
	gruel	3	2.4000	
	wild	3	2.5000	
	blood	3		3.6067
	Sig.		.903	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .052.

a. Uses Harmonic Mean Sample Size = 3.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Histidine

	Worms	N	Subset			
			1	2	3	4
Tukey HSD[a,b,c]	Water	3	1.4967			
	gruel	3		1.5967		
	wild	3			1.7967	
	blood	3				2.4067
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .001.

a. Uses Harmonic Mean Sample Size = 3.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Lysine

	worms	N	Subset			
			1	2	3	4
Tukey HSD[a,b,c]	water	3	5.6000			
	gruel	3		5.8000		
	wild	3			6.4100	
	blood	3				6.8667
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .005.

a. Uses Harmonic Mean Sample Size = 3.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Arginine

	worms	N	Subset			
			1	2	3	4
Tukey HSD[a,b,c]	water	3	4.7733			
	gruel	3		5.3833		
	wild	3			5.7967	
	blood	3				6.4000
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .003.

a. Uses Harmonic Mean Sample Size = 3.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Appendix H

Solutions and Reagents used

Preparations of the stock solutions used in this work are given below: (all the working solutions used in this work were prepared from the stock solutions).

5 M NaCl

29.22 g of NaCl was dissolved in distilled water to a final volume of 100 ml. The solution was autoclaved and stored at room temperature.

1 M KCL

7.444 g of KCl was dissolved in deionized water to a final volume of 100 ml. The solution is sterilized by filter sterilization (0.22 µm filter).

1 M $MgCl_2$

20.33 g of $MgCl_2$ was dissolved in deionized water to a final volume of 100 ml. The solution is sterilized by filter sterilization (0.22 µm filter).

1 M $MgSO_4$

24.648 g of $MgSO_4$ was dissolved in deionized water to a final volume of 100 ml. The solution is sterilized by filter sterilization (0.22 µm filter).

1 M glucose

19.817 g of Glucose was dissolved in deionized water to a final volume of 100 ml. The solution is sterilized by filter sterilization (0.22 µm filter).

0.5 M EDTA

186.1 g of $Na_2EDTA.2H_2O$ and 20.0 g of NaOH pellets were added and dissolved by stirring to 800 ml distilled water on a magnetic stirrer. The pH was adjusted to 8.0 with a few drops of 10 M NaOH and final volume was made up to 1L with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

3 M sodium acetate

40.81 g of Na_2 $(CH_3COOH).H_2O$ was dissolved in 80 ml of distilled water. The pH was adjusted to 5.2 with glacial acetic acid. The final volume was adjusted to 100 ml with distilled water and the solution was sterilized by autoclaving. It was stored at 4°C.

TAE buffer

242 g of tris-base, 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA (pH 8.0) was taken and distilled water was added to the mixture to make 1L. 1X concentrated TAE buffer was made by adding 10 ml 50X TAE buffer with 490 ml distilled water and stored at room temperature.

Ethidium bromide solution

10 µl of ethidium bromide was dissolved in 100 ml TAE buffer to make a final concentration of 20 mg/ml and stored at 4°C in the dark.

Gel loading buffer

Ingredients	Amount (g/L)
Sucrose	6.7
Bromophenol blue	0.04
Distilled water	Up to 1 L

Wizard® SV Gel and PCR Clean-Up System. Catalog No. A9282

Reagents	Purpose
Membrane Binding Solution	Help in binding of PCR product
SV Minicolumn	For Binding of PCR product
Collection Tube	For collection of flow throw
Membrane Wash Solution	For washing purposes
Nuclease-Free Water	For elution of the purified DNA from the GD column
SYBR Green master mix	

Wizard®Plus SV Minipreps DNA Purification System. Catalog No.A1460 (For Plasmid DNA)

Reagents	Purpose
Cell Resuspension Solution	For the resuspension of pelleted cells
Cell Lysis Solution	For lysis of cells
Alkaline Protease Solution	For the degradation of cellular proteins
Neutralization Solution	For Nutralizaton of AlkilineProease
Spin Column	For Binding of the plasmid DNA molecules
Collection Tube	For collection of flothrow
Wash Solution	For washing purposes
Nuclease-Free Water	For elution of the plasmid DNA from the GD column

Maxwell® 16 Total DNA Purification Kit. Catalog No. AS1050

Reagents	
Maxwell® 16 RNA Cartridges	Lysis Buffer
DNA Dilution Buffer	Clearing Agent (CAA)
Nuclease-Free Water	Mercaptoethanol, 97.4%
Clearing Columns	Collection Tubes
Plungers	Elution Tubes

ATP™ Genomic DNA Mini Kit (Blood/Culture Cell/Bacteria) Catalog No. AGB100/AGB300

Reagents	**Purpose**
GT Buffer	For the resuspension of pelleted cells
GB Buffer	For lysis of cells
GD Column	For Binding of the DNA molecules
Collection Tube	For collection of flothrough
W1 Buffer	For washing purposes
Wash Buffer (ethanol added)	For washing purposes
Elution Buffer	For elution of DNA from the GD column

Appendix H

Fasta format of COI and 16S rDNA sequence of tubificid worms

>TM_Tubifex_COI

GAGCAGGAATAGTCGGAACAGGCACTAGAATCCTAATTCGGACTGAACTAGCCCAACCTGGC
TCCTTTCTTGGAAGAGACCAACTATATAATACCTTAGTAACAGCCCACGCATTCTTAATAATC
TTCTTCATAGTAATGCCAATCTTCATCGGCGGTTTCGGAAATTGACTCCTCCCCCTTATACTTG
GGGCTCCAGATATAGCATTCCCACGAATAAACAACCTTAGATTTTGATTAATGCCCCCATCCT
TAATTCTCCTTGTATCTTCAGCCGCAGTAGAAAAAGGAGCCGGAACAGGATGAACTGTCTAC
CCGCCCCTAGCCAGTAACCTAGCCCACTCAGGACCATCAGTAGACCTGGCCATCTTCTCTCTA
CACTTAGCGGGGGCCTCATCAATTTTAGGCTCTATTAACTTCATCACTACCATAATCAACATA
CGCTCAAAAGGTATACGACTAGAGCGAATCCCACTATTCGTGTGAGCAGTTATCATTACAACT
ATTCTTCTGGTGCTAACCCTTCCTGTTTTAGCTGGTGCAATTACCATACTACTAACAGATCGAA
ACCTAAACACCTCGTTCTTCGATCCTGCGGGAGGTGGAGACCCGGTACTATATCAACACCTAT

>TJ_Tubifex_COI

GAGCAGGAATAGTGGGCACAGGAACTAGAATTCTAATTCGAACCGAACTAGCTCAACCTGGA
TCCTTCCTTGGAAGAGATCAATTATATAACACCCTAGTAACAGCTCACGCATTCCTAATAATC
TTCTTCATGGTAATACCAATCTTTATTGGTGGCTTTGGAAACTGACTACTTCCTCTTATACTCG
GAGCCCCAGATATAGCATTCCCACGAATAAACAACCTTAGATTTTGACTAATACCTCCATCCT
TAATTCTCCTTGTATCCTCAGCCGCAGTAGAAAAAGGAGCCGGAACAGGATGAACTGTATAC
CCACCACTAGCCAGAAACCTAGCCCACTCTGGACCATCAGTAGATCTAGCCATCTTCTCTCTA
CACTTAGCTGGGGCCTCATCAATTTTAGGCTCAATCAACTTTATCACCACCATAATTAACATA
CGCTCAAAAGGAATACGACTAGAACGAATCCCTCTATTCGTATGAGCAGTTATCATTACAACC
ATTCTCCTGGTACTAACCCTACCAGTTCTAGCTGGTGCAATTACCATACTACTAACAGACCGA
AACCTAAACACCTCATTCTTCGATCCTGCTGGAGGCGGAGATCCGGTACTATACCAACACCTA
T

>TD_Tubifex_COI

GGGCAGGAATAGTAGGCACAGGAACTAGACTCTTAATTCGATTTGAGCTAGCACAACCTGGG
TCATTTCTTGGCAGAGATCAACTATATAACACACTAGTTACGGCTCATGGATTTCTAATAATT
TTCTTCATAGTAATACCAATTTTTATTGGGGGCTTCGGAAACTGATTAGTACCTTTAATACTTG
GGGCTCCTGATATAGCCTTTCCACGACTTAATAATCTAAGATTTTGATTAATGCCACCCTCACT
AATCCTATTAGTCTCCTCTGCCGCAGTTGAAAAGGGGGCAGGAACAGGATGAACTGTATACC
CTCCTCTCGCTAGTAATCTGGCACATTCAGGGCCCTCTGTAGACCTGGCAATCTTCTCACTTCA
TTTAGCAGGAGCTGCATCAATTCTGGGGGCAATCAACTTCATTACTACAATAATTAATATACG
ATGAAAAGGAATACGACTAGAACGTATTCCATTATTTGTTTGATCTGTGATTATTACAGTAAT
CTTACTTCTTCTAACTCTACCAGTCTTAGCTGGCGCAATTACTATACTTCTAACAGACCGAAAC
CTAAACACTTCTTTCTTCGACCCTGCAGGAGGGGGTGACCCAGTCCTTTATCAACACTTAT

>TD_Tubifex_species 16SrDNA

AACGGCCGCGGTATCCTGACCGTGCAAAGGTAGCATAATCATTTGCCTATTAATTGTAGGCTG
GTATGAACGGATAAACGCGAACCATACTGTCTCCTCTAGAACCTTAAAAATTTATATCCAAGT
GAAGAAGCTCAAATAATCTCGCAGGACAAGAAGACCCTATAGAGCTTTACCATAAATCTTAT
CATATATACTAACTGGGTCGGTTGGGGCGACCCAGGAATTTACATCATCCTAAAACTTTAAGA
TTTACTAATCACTAAAAAGATCCTTTACAAAGATCATCGAATCAAGCTACCTTAGGGATAACA
GGCTAATTCCTCTAGAGAGCCCTTATTGACAGAGGAGGTTGGCACCT

>TM_Tubifex_species 16SrDNA

AACGGCCGCGGTATCCTGACCGTGCAAAGGTAGCATAATCATTTGCCTATTAATTGTAGGCTG
GTATGAACGGATAAACGAGAACCATACTGTCTCCTCTAGAACCATAAAAATTTATATCCGAG
TGAAGAAGCTCAAATAATCTCGCAGGACAAGAAGACCCTATAGAGCTTTACCATAAATCTTA
TCATATAAGCTAACTGGGTCGGTTGGGGCGACCCAGGAATTTACATCATCCTAAAACTTTAAG
ATTTACTAATCACTAAAAAGATCCTTTACAAAGATCATCGAATCAAGCTACCTTAGGGATAAC
AGGCTAATTCCTCTAGAGAGCCCTTATTGACAGAGGAGGTTGGCACCT

>TJ_Tubifex_species 16SrDNA

AACGGCCGCGGTATCCTGACCGTGCAAAGGTAGCATAATCATTTGCCTATTAATTGTAGGCTG
GTATGAACGGATAAACGCGAACTATACTGTCTCCTTTAGGATCTTAAAAATTTATATTCAAGT
GAAGAAGCTCAAATACTTTCGCAGGACAAGAAGACCCTATAGAGCTTTACCATAAACCTTAT
CGTATATAATAACTGGGTCGCGTGGGGGGAGACCAGAGAACACATCTCCTCCTTAATTATTA
AGATTAACTAATCTCAAAAAAGATCCTCTTTGGAGATCACAAAATCAAGCTACCTTAGGGAT
AACGGGCTAATTCCTCTAGAGAGCCCTTATTGACAGAAGAGGTTGGCTCTC