Assessment of Genetic Diversity of Zebrafish (*Danio rerio*) from Arunachal Pradesh

Dissertation submitted in partial fulfillment
of the requirements
for the degree of

M.F.Sc. (Fish Genetics and Breeding)

by

Dani Rupa, B.F. Sc.
(FGB-MA8-01)

ICAR-CENTRAL INSTITUTE OF FISHERIES EDUCATION
(Deemed-to-be University Established Under Section 3 of UGC Act 1956)
Panch Marg, Off Yari Road, Versova,
Andheri (W), Mumbai – 400 061

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“DEDICATED TO MY PARENTS”

भारतीय कृषि अनुसंधान परिषद
केन्द्रीय मात्स्यिकी शिक्षा संस्थान
(वि. अ. आ. अधिनियम धारा-3 के अंतर्गत विश्वविद्यालय)
ICAR-CENTRAL INSTITUTE OF FISHERIES EDUCATION
(University under Sec.3 of UGC Act)

Dated: 30 August, 2020

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Certified that the dissertation entitled " **Assessment of Genetic Diversity of Zebrafish (*Danio rerio*) from Arunachal Pradesh**" is a record of independent bonafide research work carried out by **Ms. Dani Rupa** during the period of study from August, 2019 to September, 2020 under our supervision and guidance for the degree of **Master of Fisheries Science (Fish Genetics and Breeding)** and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

Advisory Committee

Major Advisor

(Dr. Mujahidkhan. A. Pathan)
Scientist, FGB Division
ICAR-CIFE, Mumbai-61

(Dr. Aparna Chaudhari)
Principal Scientist & Head
FGB Division,
ICAR-CIFE, Mumbai-61

(Dr. A. Pavan Kumar)
Senior Scientist
FGB Division,
ICAR-CIFE, Mumbai-61

(Mr. Angom Lenin Singh)
Scientist
FGB Division
ICAR-CIFE, Mumbai-61

पंचमार्ग, ऑफ यारी रोड, वरसोवा, अंधेरी (प.) मुंबई - 400 061
Panch Marg Off Yari Road,
Versova, Mumbai – 400 061

Tel. No. (Office) : 022-26361406/7/8,
Fax: 91-022-26361573
http://www.cife.edu.in

DECLARATION

I hereby declare that the dissertation entitled "**Assessment of Genetic Diversity of Zebrafish (*Danio rerio*) from Arunachal Pradesh**"is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

(Dani Rupa)
M.F.Sc. Student
ICAR-Central Institute of
Fisheries Education

Date: 30th August, 2020
Place: Mumbai

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सार

ज़ेब्राफिश, डैनियो रेरियो, जेनेटिक्स, इम्यूनोलॉजी, डेवलपमेंट और कैंसर बायोलॉजी आदि से संबंधित अध्ययनों के लिए एक उत्कृष्ट कशेरुकी मॉडल जीव के रूप में उभरा है, हालांकि ज़ेब्राफ़िश के प्रयोगशाला उपभेदों पर गहन शोध किया जाता है, जंगली ज़ेब्राफिशिश के संबंध में दुर्लभ जानकारी उपलब्ध है। उपभेदों, जनसंख्या का आकार और संरचना। अरुणाचल प्रदेश में ज़ीरो घाटी एक सुरम्य घाटी है जो समुद्र तल से 1573 मीटर की ऊँचाई पर स्थित है। इस घाटी में धान-सह-मछली की खेती लोकप्रिय है और सिंचाई के लिए उपलब्ध पानी नदियों को जोड़ने वाली नहरों से । धान के खेतों को जोड़ने वाली ये सिंचाई नहरें ज़ेब्राफिश के लिए मुख्य केंद्र बनाती हैं। वर्तमान अध्ययन में जीरो घाटी के तीन अलग-अलग स्थलों से लगभग 61 वयस्क ज़ेब्राफिश नमूने एकत्र किए गए थे। लगभग 12 मॉर्फोमेट्रिक पैरामीटर सेक्स वार ज़ेब्राफिश में दर्ज किए गए थे। कुल लंबाई, मानक लंबाई, दुम पेडुंल लंबाई, शरीर की अधिकतम गहराई, पूर्व-संचालन की लंबाई, गुदा के आधार की लंबाई और पृष्ठीय पंख की लंबाई के लिए एलएस का अर्थ है 3.88 6 0.06 सेमी, 3.14 ± 0.05 सेमी, 0.35 ± 0.01 सेमी, आदि। पुरुष zebrafish की तुलना में क्रमशः 0.86, 0.01 सेमी, 0.58 ± 0.01 सेमी, 0.51 and 0.01 सेमी और 0.50 cm 0.01 सेमी और काफी अधिक थे। सेक्स का प्रभाव zebrafish में कुल लंबाई, मानक लंबाई और शरीर की अधिकतम गहराई पर महत्वपूर्ण था। ज़ीरो घाटी से एकत्र ज़ेब्राफिश में आनुवांशिक विविधता का मूल्यांकन छह माइक्रोसेलेलाइट लोकी अर्थात Z20576, Z7141, Z7156, Z10215, Z20966, Z15457 का उपयोग करके किया गया था। Z20576 ट्रिन्यूक्लियोटाइड था और बाकी डाइन्यूक्लियोटाइड थे। Locus Z20966 मोनोमोर्फिक था। कुल मिलाकर मनाया गया विषमलैंगिकता, मार्करों के लिए अपेक्षित विषमता और पीआईसी क्रमशः 0.46, 0.61 और 0.62 था। ज़ेब्रिफिश स्टॉक के बेहतर लक्षण वर्णन के लिए ज्यामितीय आकारिकी जैसे और अध्ययन किए जाने की आवश्यकता है।

ABSTRACT

The zebrafish, *Danio rerio*, has emerged as an excellent vertebrate model organism for studies related to genetics, immunology, developmental and cancer biology, etc. Even though laboratory strains of zebrafish are subjected to intense research, scarce information is available with respect to wild zebrafish strains, population size and structure. Ziro valley in Arunachal Pradesh is a picturesque valley that lies at an altitude of 1573 meter above mean sea level.Paddy-cum-fish cultivation is popular in this valley and the water available through irrigation canals connecting the streams. These irrigation canals connecting paddy fields form the main hub for zebrafish. In the present study about 61 adult zebrafish samples were collected from three different sites of Ziro valley. About 12 morphometric parameters sex wise were recorded in zebrafish. The LS means of female zebrafish for total length, standard length, caudal peduncle length, maximum body depth, pre-opercular length, anal fin base length and dorsal fin length were 3.88 ± 0.06 cm, 3.14 ± 0.05 cm, 0.35 ± 0.01 cm, 0.86 ± 0.01 cm, 0.58 ± 0.01 cm, 0.51 ± 0.01 cm and 0.50 ± 0.01 cm respectively and were significantly higher when compared to male zebrafish. The effect of sex was significant on total length, standard length and maximum body depth in zebrafish. The genetic diversity in zebrafish collected from Ziro valley was assessed using six microsatellite loci viz., Z20576, Z7141, Z7156, Z10215, Z20966, Z15457. The Z20576 was trinucleotide and the rest were dinucleotides. The locus Z20966 was monomorphic. The overall observed heterozygosity, expected heterozygosity and PIC for the markers was 0.46, 0.61 and 0.62 respectively. Further studies like geometric morphometry need to be conducted for better characterization of zebrafish stocks.

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INTRODUCTION

1. INTRODUCTION

The zebrafish, *Danio rerio*, teleostei infraclass, cyprinidae family, is a monophyletic group that is thought to have arisen approximately 340 million years ago from a common ancestor (Meyer *et al*., 1999). It has emerged as an excellent vertebrate model organism for studies related to genetics, immunology, developmental and cancer biology, etc. Zebrafish is widely used for modeling of human diseases because of genetic homology, physiology, and developmental similarity with humans (Bakkers, 2011, Liu and Stainier., 2012). Even though laboratory strains of zebrafish are subjected to intense research, scarce information is available with respect to wild zebrafish population size and structure. Zebrafish has emerged as an excellent vertebrate model organism owing to external fertilization and development, optical clarity during embryogenesis, high fecundity, short generation times and ease of maintenance (Kimbery and Leonard 2000; Zhao *et al.,* 2015). Over 400 laboratories worldwide and around 50 labs in India conduct research with zebrafish. Globally, about 18 'wild-type' zebrafish lab strains have been established (Spence et al. 2008). This represents only a tiny fraction of natural genetic diversity of zebrafish. Most commonly used strains are AB, TL, HL, WIK, and SJD and they have undergone several rounds of close related mating. In future, there is need for more number of genetically characterized strains to sustain zebrafish research. Such strains developed will have a global demand.

The zebrafish is native to freshwater habitats in South Asia, where it is found in India, Pakistan, Bangladesh, Nepal, and Myanmar (Lawrence*,* 2007). In India, zebrafish is found in Brahmaputra, Gandak, Rapti, Karnal, Kali, Ramganga, Ganges, Yamuna, and Indus river drainages, Nadhave and Kalauma rivers in Kumaon Himalayas, Balangi district, Orissa Western Ghats, Kabini River, Thunga River and Wynaad district of Kerala (Menon., 1962, Hora., 1937, Chauhan., 1953 andTilak., 1968., Parichy, 2015). The zebrafish have been reported at elevations of ~8–1576m and in a range of water conditions, including temperatures between 12–39˚C, pH levels of 5.9–9.8, and salinities of ~0.01–0.8 (Spence *et al*., 2006; Engeszer *et al*.,

2007; Arunachalam *et al*., 2013). It typically inhabits the moderately flowing top stagnant clear water of quite shallow depth in streams and canals. As the zebrafish are distributed over a wide geographical range, there is a strong possibility that major phylo-geographic breaks occur (Whiteley *et al.,* 2011) and there is need to assess it. Genetic diversity within species (between populations as well as among individuals within populations) as part of biodiversity is the result of the degree of variation at different levels (nucleotide, gene, chromosome, and genome). The presence of genetic variation plays an important role in species/population survival and in their successful evolutions in response to both short-term and long-term environmental changes (Dudu *et al.,* 2015).The molecular markers such as microsatellite markers which consist of multiple copies of tandemly arranged simple sequence repeats (SSRs) that range in size from 1 to 6 base pairs (Cordes*,* 2004) are useful for assessment of genetic diversity. They are useful in population genetic analysis for quantifying genetic variation within and between the population of species and for stock identification as well as inter-population comparison (Munner, 2014). The word 'Morphology' is derived from the ancient Greek word "Morphe" meaning form and "logos" meaning the study. Morphology is a highly integrative trait that combines functional and evolutionary information, and morphometrics is the statistical description of shapes, which is further distinguished as traditional morphometric and modern morphometric Traditional morphometric depends on the measurements of shape indicators such as lengths, areas, angles, and ratios, whereas modern morphometrics considers the entire shape, configurations of landmarks, and outline analysis (Caillon *et al.,* 2018). Morphometric characters are the measurable characters of a fish and are the simplest and direct method of fish identification and diversity. It identifies the differences between the same species of different geographical regions and contrasting morphometric characters describes the evolutionary adaptations. The use of geographic morphometry in zebrafish will be effective in distinguishing sex, geographical ranges, and strains for ecological and phylogenetic studies of fish populations.

Arunachal Pradesh is located between 26.28°N and 29.30°N latitude and 91.20°E and 97.30°E longitude and has an area of 83,743 square km. It is also known as the land of Dawlit mountains as its land is first touched by the rays of the sun in the Indian soil. Arunachal Pradesh is the easternmost state of India and is a micro hotspot within the larger eastern Himalayas. The state is one of the global biodiversity hotspots and is endowed with rich flora and fauna (Singh *et al.,* 2010). Records on fish were first reported by McClelland during the early 1800s when he described four fish species from the state, and then Chaudhuri (1911- 1913) reported 21 fish species from his Abor expedition during 1911-1912. At present, a total of 259 fish species under 105 genera, 34 families, and 11 orders of which 32 species are endemic in the state and 19 species are considered threatened as per IUCN. The state has huge fisheries potential as it harbors commercially important food, sports, or ornamental fishes (Gurumayum *et al.,* 2016).The valley in Arunachal Pradesh is a picturesque valley that lies at an altitude of 1573 meter above mean sea level. This valley is situated in the central part of Arunachal Pradesh in the Lower Subansiri district. The Ziro valley is also known as the Apatani valley as the "Apatanis" are the original inhabitants of this valley. Paddy-cum-Pisciculture was adopted during 1965-1966, and it is believed that "Apatanis" were the first to have known of paddy-cum-fish culture in India (Reena & Anku, 2014). About 48.38% of land comes under paddy-cum-fish cultivation (Kacha, 2016) where water for irrigation is made available through irrigation canals connecting the streams. These irrigation canals connecting paddy fields form the main hub for zebrafish. The valley is thus a rich resource of wild type zebrafish and if genetically characterized and maintained in the captivity, it may cater to needs of various zebrafish researchers. With this background, the following objectives have been proposed.

OBJECTIVES

- To study variation in morphometric traits of zebrafish (*Danio rerio*)
- To assess genetic diversity in zebrafish (*Danio rerio*) using microsatellite marker.

REVIEW OF LITERATURE

2. LITERATURE REVIEW

2.1. Importance of Zebrafish as a model organism

Zebrafish (*Danio rerio*) is first described in a book about fishes of Ganges by English Physician Sir Francis Hamilton (Hamilton 1822). It is a prominent model organism in biological research in recent times. It was first used as a model by George Streisinger (university of Oregon) in the1970s because it was simpler over the mouse and easy to manipulate genetically (Khan & Alhewairini., 2018). Zebrafish is a powerful model organism for research, over the last 40 years into vertebrate genetics (J.R. Meyer, 2018). The sequenced genome of zebrafish obtained (Biga & Goetz. 2006) was used to compare genomic characteristics of zebrafish with the human reference population and other Zebrafish lines, which have helped to relate the data with the mammalian system (Troung *et al*., 2014). The salient feature of zebrafish are external fertilization, high fecundity vertebrate animal, tremendously faster growth rate, cheaper maintenance in the lab, and its exceptional ability to repair and regenerate muscle. Zebrafish an ideal model organism in biological research (Volker *et al.,* 2007; Lammer, 2009; Oliveira *et al.,* 2009) as many gene functions are similar between fish and humans, the understanding on how cells and tissues work in this fish translated into new methods for researching and understanding a broad range of human diseases. Thus, the zebrafish are used for studying gene, gene expression and function, cellular growth and migration, inflammation, regeneration of tissues, pigmentation, blood clotting, vascular development, heart disease, retinal disorders, hearing disorders, musculoskeletal conditions, neurodegenerative diseases, movement disorders, psychiatric and behavioral disorders, cancer, and gene and drug therapies (Holtzman, 2007).

Many zebrafish strains are available, such as AB, TU, TL and WIK. The laboratory strains *viz*; TU, AB, and WIK, are all considered common wild-type laboratory strains and differ morphologically, genetically, physiologically and behaviorally. They have different timelines and "geographical" origins. TU originated from several stocks from a pet shop in Tübingen (Germany) in 1997. AB originated from crosses from two pet shop stocks in Albany. These latter strains can be

purchased from, e.g., ZIRC or EZRC that maintains supplies of these strains (Oregon, USA) as early as 1970. Mitochondrial DNA analysis suggests that AB originated from the Ganges/Brahmaputra region near Calcutta (Kolkata) in India (Whiteley *et al*., 2011). WIK originated from a single-pair mating of wild-caught fish in India (Calcutta) in 1997 (Holden and Brown, 2018; Rauch *et al*., 1997). Besides, AB underwent two rounds of gynogenesis, with six pairs being the founders of subsequent AB zebrafish. They are cleaned up to remove lethal embryonic mutations. Finally, the TL strain contains two mutations, making it different in physical appearance: it is homozygous for leot1 (a recessive mutation causing spotting in adult fish) and for lofdt2 (a dominant mutation causing long fins). The commonly used laboratory strain had a low level of genetic variability. The fish collected from different sources but belonging to similar strains may differ in their levels of genetic variation as in AB strain(Coe *et al*.,2018) and may be considered bolder than wild populations (Drew *et al*., 2012; Oswald and Robison, 2008; Wright *et al*., 2006). When it comes to sex determination/differentiation, wild populations, as well as the laboratory strain WIK, have a locus on chromosome 4, which determines their sex (homozygous: males; heterozygous: mostly females), while this locus is lost in the laboratory strains AB and TU (Holtzman *et al*., 2016; Wilson *et al*., 2014). In AB and TU, sex determination/differentiation seems to occur by interplaying yet another set of genes and factors in the environment (Holtzman *et al.,* 2016; Liew and Orban, 2013). Factors such as high density, high temperature, low food availability, and hypoxia have shown to bias sex ratios toward males (Holtzman *et al.,* 2016). AB and TU may thus be more sensitive to these factors as it comes to sex determination/differentiation than wild fish. Finally, laboratory strains have increased growth rate, weight, and fat content for, which a QTL found on chromosome 23 (Oswald and Robison, 2008; Wright *et al.,* 2006)

2.2. Strains available and Zebrafish Resources

Genetic variation may be found within laboratory strains, WIK has, as expected, more genetic variation than AB, TU, or TL (Butler *et al.,* 2015; Coe *et al.,* 2009; Rauch *et al.,* 1997). Yet, the number and length of CNVs are higher in TU than in WIK or AB (Brown *et al.,* 2012). This may be due to the large founding size and composite population origin of TU in relation to the other two strains (Brown *et al.,* 2012). Also, differences occur between TU, AB, and WIK, as shown in liver mRNA expression profiles (Holden and Brown, 2018). For instance, while a wide variation in sex-specific gene expression exists in AB and WIK, this does not occur in TU (Holden and Brown, 2018). Some genes were more strongly or less firmly expressed in one strain or another (Holden and Brown, 2018; Whiteley, 2011). Furthermore, there is substantial genetic variation between laboratory populations of the same strain (Brown *et al.,* 2012; Butler *et al.,* 2015; Coe *et al*., 2009**)**. No zebrafish strain exists that fulfills these criteria, but C32 (91% homozygous), SJD (90% homozygous), and IM (95% homozygous) are considered to be the most "isogenic" or "inbred" zebrafish strains (Nechiporuk *et al.,* 1999; Shinya and Sakai, 2011).There is a difference in individual motion and shoaling tendency between strains of the same species. Strains AB and TL were selected to study shoaling activity. In an experimental tank, both the strains avoided free space and preferred to swim near the walls of the tank and landmarks. The AB strains are more prone to the vicinity than TL, and TL is more prone to form the shoaling pattern. Thus, different strains of the same species show different behavior in the same environment (Seguret *et al.,* 2016). The same strain of zebrafish domesticated in various facilities will be genetically different *i.e*., same strains of zebrafish kept in separate facilities will evolve in distinct substrain studied using microsatellite in AB and WIK strains (Suurvali *et al.,* 2019).

The Sequence Read Archive at the NCBI has ~60,000 accessions for zebrafish, and other major databases such as ZFIN (Zebrafish Information Network) are available (Butler *et al*., 2015; Howe *et al.,* 2017; Gutierrez *et al*., 2019), which represents the most massive volume of data collected from any fish species to date.

More than half of the ~34,000 PubMed-listed research articles on zebrafish appeared after publication of the reference genome (Howe *et al.,* 2013), based on the Tübingen strain (TU) initially obtained from a pet store in Tübingen, Germany, with no further information on the origin (Haffter *et al.,* 1996). Besides, many internet links assist those interested in zebrafish as a teaching and research tool such as online publications, specific information on zebrafish, and Zebrafish journals, associations, and societies. Some of the Zebrafish resources are zebrafish International Resource Center (ZIRC), Zebrafish-National BioResource Project, Japan, Norwegian Zebrafish platform Research council of Norway, European Zebrafish Resource Center (EZRC), National Zebrafish Resources of China, Latin American Zebrafish Network (LAZEN), TaiwanZebrafish Care Facility at Academia Sinica (TZCAS) and Taiwan Zebrafish Care Facility at ZeTH. These resources are used as a preliminary guide for locating the information that will aid in search for methodology, techniques, and organization associated with Zebrafish (Stephen & Smith., 2012).

2.3. Taxonomy of *Danio rerio* (F. Hamilton, 1822)

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Cypriniformes

Family: Danionidae

Genus: *Danio*

Species: *rerio*

2.3. Biology

Danio rerio is a small-sized teleost fish, and adult reaches a length of about 4 to 5cm. Their body is cylindrical in shape, and a distinct color pattern with altering light and dark horizontal stripes, therefore they are commonly called as zebrafish.

They are sexually dimorphic, where males are thinner and generally golden in the ventral region whereas, females are more rounded and silvery, mainly in the ventral origin, and have a distinct genital papilla. They are oviparous species and has an average fecundity of 1500 to 1800.Light and temperature are the important factors that have an influence on breeding and hatching of eggs. Even, a slight increase in temperature facilitates breeding. Breeding tanks should be maintained at 26°C. Light accelerates the hatching of eggs and also influences meristic character. A significant variation was found in head length and standard length and also variation was found in the number of vertebrae and anal fins in zebrafish which developed in light and in darkness (Laale,1975). The fry grows quickly and can reach sexual maturity within 2-3 months (Simonett *et al.,* 2016). Zebrafish have a terminal mouth and omnivores feeding habit, observations of gut contents and inspection from almost all habitat types where the species occurs state that zebrafish feeds primarily on allochthonous materials, usually ants and other insects falling into streams, secondary channels, and pools (Arunachalam *et al.,* 2013).

2.4. Geographical distribution: World

In the wild, *Danio rerio* is a tropical freshwater fish living in small rivers, streams, paddy fields, and channels in South Asia, including India, Myanmar, Bangladesh, and Nepal (Lawrence*.,* 2007). Zebrafish prefers low-flow waters with vegetative overhangs that end with few predators(McClure *et al.,*2006; Spence *et al.*, 2006; Engesser *et al.,* 2007; Arunachalam *et al.*, 2013), though they are occasionally found in deeper running streams, usually in inlet regions (Arunachalam *et al.,* 2013). More massive streams are possible avenues for the movement of fish between isolated populations, thus increasing genetic diversity.

2.5.1. Geographical distribution: India

In India, Zebrafish are found in streams and canals of the Sutlej, Brahmaputra, Chindwin river basins, Western Ghats and the Eastern Ghats, Gandak, Rapti, Kali, Ramganga, Ganges, Yamuna and Indus river drainages, Nadhave and Kalama

rivers in Kumaon Himalayas, Balangri district, Orissa Western Ghats, Kabini River, Thunga River, and Wayanad district of Kerala (Hora, 1937; Chauhan and Ram Krishna, 1953; Menon, 1962; Tilak, 1968). Some of the region in North-East India where zebrafish can be found are Mawlymkhang Umsingh Highway, Meghalaya, Subansiri River at Arunachal Pradesh,Dhalpur stream at Bipuria town Assam, Ghagor stream at Ghagor Assam, Dikrong River at Khola camp Arunachal Pradesh, Rani village at Siang, Arunachal Pradesh and Sora River at Hapoli Village, Arunachal Pradesh. Zebrafish were found from beels and household fish ponds of Brahmaputra River, inlets and outlets of culture ponds where there is an exchange of water. Also, in the paddy field which is connected to low land streams or irrigation canals where they make its habitat to the areas where water overflows into the channel/stream. Low-flow, shallow habitats were the preferred habitats of *Danio rerio*. Zebrafish were distributed within a wide range of pH and temperature with a pH of 6.2 in Arunachal Pradesh to 9.8 in Orissa. Temperature varying from 12.3°C in the high terrains of Arunachal Pradesh to a peak temperature of 28.4° C in the summer of Uttar Pradesh at River Muthumathi. In general, in this region, *Danio rerio* utilised a wide range of habitats across the countryside and hence was widely distributed (Arunachalam *et al.,* 2013).

2.5.2. Arunachal Pradesh: Geography and Biodiversity

Arunachal Pradesh is known as the land of Dawnlit mountains as Dong in Anjaw district is the first place on the Indian mainland to witness the first sunrise also the Orchid state of India. It is present in the extreme north-eastern corner of India and is the largest state among the entire North-eastern states encompassing an area of 83,743 km^2 and sharing its boundary with the neighboring countries, Bhutan in the west (160 km), China in the north (1030 km) and Myanmar in the east (440 km). There are 25 districts with 26 major tribes and ten sub-tribes and numerous minor tribes. Blessed with varied landscapes and forests, 96 percent of the landscape is of hilly terrain. Around 80 percent is covered by the forest, which has been why the state experiences diverse climatic conditions and natural boundaries for the different communities. Forest in Arunachal Pradesh is classified as alpine

forest, temperate forest, subtropical forest, and semi-evergreen forest. Dense forests and giant trees are considered to be an ancestral soul by the localities. Grasslands, as well as riverine grasslands, cover mountains. Wetlands in the state are the home to various fish species and wildlife, including threatened and endangered species. Numbers of rivers and streams flow through the hilly terrain. Some of the significant Rivers draining the area are Dibang, Kamla, Kamplang, Lohit, Nao-Dihing, Siang, Siyum, Subansiri, and Tirap, which divides the states into five different river valley: the Kameng, the Subansiri, the Siang, the Lohit and the Tirap. The mightiest of all the above River is the Siang, popularly known as Tsangpoin Tibet, joins with Dibang and Lohit in Assam and forms the Brahmaputra. Arunachal Pradesh is one of the eighteenth "Biodiversity hotspots" in the world. The state is rich in agro-biodiversity, bearing 5000 species of flowering plants, out of which 238 are endemic to the state, more than 500 species of fauna and is a shelter to four significant cat, tiger, leopard, clouded leopard and snow leopard, and rare lesser feline species like the Golden cat and marbled cat. Although substantial natural sources are available, practices such as jhum cultivation, encroachment problem, and urbanization, agriculture expansion, development of pastoral lands, hydro-power project causing habitat destruction of threatened and endemic flora and fauna have affected the nature immensely. Establishment of seven wildlife sanctuaries, one Orchid sanctuary, two National parks, a protected area, reserve forest and setting up rules by the government like fishing ban during breeding season and prohibition on hornbill hunting, the state animal of the state are some of the initial steps taken by the government to protect the potential resource of Arunachal Pradesh.

2.5.3. Arunachal Pradesh: Fish Diversity

Arunachal Pradesh is the largest state amongst other north-eastern states with an area of. It has the largest river drainage area in North-Eastern India. Both lotic water bodies like rivers, streams, lakes, drains and lentic water bodies like wetlands, which covers an area of 1,34,244 ha are present from lower to higher altitudinal areas and mostly drains in the the river Brahmaputra and others in the Irrawaddy River drainage of Chindwin basin (Gurumayum and Tamang, 2017). The occurrence

of fish in the state was first reported during the 1800s when McClelland reported four fish species, 21 fish species reported by Chaudhuri (1911-1913) during his Abor expedition, and many such reports of the existence of different fish species from various other water bodies (Gurumayum *et al.,*2016). The first museum of fish resources established at the center of Biodiversity in RGU, Rono hills, to form a bridge to assess information regarding the list of fish fauna in the state (Bagra *et al.,* 2009). The state's diverse climatic condition forms an abode for numerous fish species and includes a biological hotspot region. It has an immense fisheries potential until now a total of 259 fish species under 105 genera, 34 families, and 11 orders are found, of which 32 fish species are considered endemic to the state and five fish species endangered, 14 fish species vulnerable, 22 threatened species as per IUCN (Gurumayum *et al.,* 2016). Large numbers of fish belonged to *Cyprinidae, Nemacheilidae, Anabantidae, Badidae, Sisoridae, Ambassidae, Channidae, Gobiidae, Clupeidae, Heteropneustidae, Bagridae, Cobitidae, Synbranchidae, Notopteridae, Nanidae, Tetraodontidae, Osphronemidae, Belonidae* families (Das *et al.,* 2013*).*Most fish are cold water hill stream fish (Bagra and Das, 2010). All the fish reported having economic importance as food, game, or ornamental fish.

People of the state, like other North-Eastern states, have the belief that all fish are meant to be eaten and only the preference of one fish over the other exits. Angling opportunities in the state have become a ray of hope for developing sports fisheries in the state. Places like Bhalukpong and Tipi on the River Kameng, Pasighat on the River Lohit, and Tezu on the River Lohit offer fishing opportunities for trout and Mahseer. The state is also a repository of ornamental fishes and one of the major contributors to the lucrative trade (Guruyum 2010). The state is mostly known for its cold water fisheries practiced in Tawang and West Kameng district where trout farming was encouraged by ICAR-Directorate of Coldwater Fisheries Research (ICAR-DCFR), Bhimtal (Baruah *et al.,* 2019), and paddy-cum-fish practiced in Ziro valley, lower Subansiri district where common carp is cultured along with rice (Reena and Anku, 2014). Das (2019) discovered five new species, and recently a new species, *Schizothorax sikusirumensis*, was found and collected from

the junction of river Siku near the Gakang area under the mebo circle of East Siang district. Due to dense rainforest, steep terrains, and communication problems, remote water bodies are not accessible to researchers, and hence all the fish species available cannot be accessed.

2.5.4. Arunachal Pradesh: Zebrafish Diversity

Restriction site-associated DNA sequencing (RAD-seq) performed from three major lineages on wild-caught zebrafish and a laboratory zebrafish. A large dataset from RAD-seq data of these two groups of fish. Both the datasets were combined and made a comparative study. A significant difference in heterozygosity and the allele frequency observed within and between these two groups. The whole-genome sequence of one wild- zebrafish from North-east India revealed nearly seven million differences (5.2 million single-nucleotide and substitutions and 1.6 million indels) from the reference genome, which states that zebrafish strains obtained from North-East India were genetically closest with the standard laboratory strains. Wild fish from Bangladesh were found to be more diverse than the laboratory strains AB, TU, EKW, WIK, and TL based on the study using microsatellites. Hence, there is a genetic difference within the laboratory fish and between the laboratory fish and the wild fish. Perhaps, due to the influence of the environment. For example- in zebrafish facilities, sanitization is done for the embryo's, standardized and regular feeding is practiced, and pathogen contact is minimized by following strict procedures and protocols. Laboratory strains also undergo genetic bottleneck, followed by different patterns of inbreeding. None of this practice followed in the natural environment for the wild-strains. Hence, laboratory strains can differ genetically among each other and with the wild-fish. Moreover, the conclusion drawn from a single laboratory strain cannot necessarily be similar to that of other strain nor fully represent the wild population. The same strains from different laboratories differ genetically; thus, they can be considered sub-strains (Suurvali *et al*., 2019).

Toxicity study on lab strains, AB (from Harvard), AB (S), TE, and TL, showed low genetic variation than the wild type. The same strain of fish from different

sources differs in levels of genetic variation as the AB strain. Genetic variation of the exposed population had influenced by exposure to toxicants (Coe *et al.,* 2008).Copy number variants (CNVs) represent genomic variation invertebrate and of genetic substructuring between the zebrafish strains. CNVs are higher in the native population than in lab strains, and larger CNVs have more sequence thus are potentially more deleterious. Three lab strains AB, WIK, and TU, and native population from Bangladesh were selected to identify and characterize CNV. CNVs were eliminated from natural people and maintained in lab strains. Thus, the highest genetic variation found in TU lab strains, and the native population from Bangladesh had lower genetic variation than TU lab strains. High genomic variation in lab strains may be due to inbreeding or relaxed selective pressure (Brown *et al*., 2011).

The zebrafish population collected from four different streams of Arunachal Pradesh, namely, Dikrong river at Khola camp, third-order streams of Rani village, Siang, and Thore Siang, and Sora River, Hapoli Village, Ziro. They were mostly available in habitat with good vegetation cover and shallow depth and width of the stream. Zebrafish mainly were collected with other fish species such as *Rasbora rasbora, Devario devario, Barillius bendelisis, Puntius sophore, Puntius conchonius and Glypthothorax species.* Zebrafish reported from Sora River, Hapoli had a more in-depth body when compared with the population of zebrafish available in other regions (Arunachalam *et al.,* 2013).

2.6. Morphometry- Importance and Methods

The word morphology is derived from the ancient Greek word "Morphe," meaning form and "logos," meaning the study. Morphology is a highly integrative trait that combines functional and evolutionary information, and Morphometrics is the statistical description of shapes and is further distinguished as Traditional morphometrics and Modern morphometrics. Traditional Morphometrics depends on measuring shape indicators such as lengths, areas, angles, and ratio, whereas modern Morphometrics considers the entire shape, configurations of landmarks, and outline analysis (Caillon *et al.,* 2015). Morphometric characters are the measurable characters of a fish and are the simplest and direct method of fish identification and

diversity. Morphometrics is defined as statistical procedures for analyzing variability in the size and shape of organs and organisms. Some points on the fish body are arbitrarily selected, known as landmarks, through which identification of individual fish shape can be assessed. In Fishery science, Morphometrics important to differentiate the fish population, to identify the difference between fish population, the difference among the same group of fish, and between species of similar species. The difference in Morphometrics among stocks of species is crucial to distinguish the population structure and identify stocks (Mojekwu & Anumudu, 2015). Methods for morphometric measurements include sample collection from the sampling sites, then anesthetizing the fish samples using Benzocaine (ethyl-p-amino benzoate) (Mojekwa & Anumudu, 2015), sex of the fish is identified based on the traditional morphometric study. Samples were digitized by Samsung SM-M307F mobile phone rear camera (48-megapixel primary sensor with an f2.2 aperture) in JPEG format with a resolution of 3264×1836 pixels; images were saved in JPEG file format and analyzed in ImageJ software, developed at the National Institutes of Health by Wayne Rasband and is a Java-based open-source image processing program. Statistical Analysis Software (SAS; version 9.3) was used for Statistical analysis. The data initially tested for normality, and the outliers were removed. The least-square means and the descriptive statistic sex-wise was estimated by invoking the PROC means, PROC univariate, and PROC GLM procedure of SAS

2.7. Types of Molecular markers

Genetic markers are classified into two categories: type I and type II. Type I are markers associated with genes of known function, while type II markers are associated with anonymous genetic segments (O'Brien, 1991). According to this classification, allozyme, ESTs, barcodes of species based on cytochrome c oxidase I (COI), and most RFLP markers are type I markers whereas RAPD, AFLP, Microsatellite markers are examples of type II markers. Transmission and evolutionary dynamics form the basis for the classification of genetic markers (Park and Moorey, 1994). In aquaculture, different genetic markers have been found useful in various aspects. Different markers have other properties and

characteristics, ease of application, abundance in the genome, and polymorphic information (PIC) content based on the preference the markers of choice can use in genetic diversity study in zebrafish. The detection of polymorphism in a population by assingning a value to a marker is referred to as PIC, and it depends on the number of detectable alleles and the distribution of their frequencies (Liu and Cordes, 2004).

2.7.1. Allozymes

The term Isozymes refers to multiple biochemical forms of the enzyme having identical substrate specificity within the same organism. They are functionally similar and separable forms of enzymes encoded by one or more loci. Allozymes or allelic isozymes are the different allelic forms of the same enzymes encoded at the same locus. Allozymes are encoded by a single gene locus and form the protein products of genes (Huseyinkucuktas and Zhanjiang Liu, 2007). Allozymes are more likely to be associated with altered phenotypes. Different allelic forms of the same gene are called allozymes, and various genes whose products catalyze the same reaction is called isozymes. An essential feature of allozyme is that they are polymorphic and have co-dominant nature of inheritance of gene products; thus, genetic interpretation of the phenotype facilitated because all products are generally visible and not masked by the dominance of one over another (Krishnan, 2013). Since the 1960s, the most commonly employed molecular method in fishery genetics is the starch gel electrophoresis of allozymes (Hillis *et al*., 1996). When a starch gel is subjected to an electrical field, the protein products begin to migrate because of their difference in their size and charge at different rates. Any variation in the presence/absence and relative frequencies of alleles are helpful to quantify genetic variation and differentiate among genetic units at the levels of populations, species, and higher taxonomic designations. Allozyme electrophoresis is commonly used in aquaculture for stock identification, parentage analysis, inbreeding analysis, and limited genetic mapping (Liu and cordes 2004). Allozymes have found application in aquaculture to track inbreeding, identify stocks, mix stock analysis, analyze parentage, and genetic

monitoring at the hatchery populations by establishing a monitoring program. Major drawbacks associated with allozymes include heterozygote deficiencies due to null alleles (enzymatically inactive) and the requirement of large and fresh amounts of tissue samples. In addition, some changes in the DNA sequence. Moreover, a number of allozyme loci and polymorphism is low.

2.7.2. Mitochondrial DNA marker

Mitochondrial DNA (mtDNA) is the DNA located in mitochondria. Studies on vertebrates have shown that sequence divergence accumulates more rapidly in mitochondrial DNA than in nuclear DNA (Brown, 1985) and attributed to a faster mutation rate in mtDNA that may result from a lack of repair mechanisms during replication (Wilson *et al.*, 1985) and a smaller effective population size due to the strictly maternal inheritance of the haploid mitochondrial genome (Birky *et al*., 1989). mtDNA is characterized by the non-mendelian mode of inheritance and is considered to be a single locus in genetic investigations (Avise, 1994). As mtDNA is maternally inherited, the phylogenies and population structure derived from mtDNA data may not reflect those of the nuclear genome due to gender-biased migration (Birky *et al.,* 1989) or introgression (Chow and Kishino, 1995). Mitochondrial markers are quite popular among aquaculture geneticists, in part due to their use in the identification of broodstock (Benzie *et al.,* 2002) and used extensively to investigate stock structure in a variety of fishes, including eels, bluefish, red drum, snappers, and sharks. In the early days of molecular analysis, the high levels of mtDNA polymorphism relative to allozymes exploited in aquaculture genetics for population differentiation. Although mtDNA loci can exhibit many alleles per locus, the limited number of markers available on the mtDNA molecule positions its PIC value higher than that of allozymes but lower than highly variable nuclear markers such as RAPD, microsatellites, AFLP, and SNPs.

2.7.3. Restriction Fragment Length Polymorphism (RFLP)

RFLP markers are a technique that exploits variations in homologous DNA sequences, known as polymorphisms, which makes identification of individuals, populations, or species or to recognize the locations of genes within a sequence. RFLP marked the beginning of an entirely new approach in genetic polymorphism (Dodgson *et al*., 1997). Specific 4, 5, 6, or 8 base pairs (bp) nucleotide sequences are recognized by restriction endonucleases, which are bacterial enzymes that cut DNA wherever these sequences encountered so that changes in the DNA sequence due to indels, base substitutions, or rearrangements involving the restriction sites can result in the gain, loss, or relocation of a restriction site. DNA fragments differ in number and size among individuals, populations, and species when restriction enzymes play their role in digesting DNA. Traditionally, Southern blot analysis (Southern, 1976) was used to separate fragments. Genomic DNA is digested and subjected to electrophoresis through an agarose gel, then transferred to a membrane, and visualized by hybridization to specific probes. The most recent analysis replaces the tedious Southern blot method with techniques based on the Polymerase chain reaction (PCR). If flanking sequences are known for a locus, the segment containing the RFLP region is amplified via PCR. If the length polymorphism is caused by a relatively large nucleotides (> 100 bp depending on the size of the undigested PCR product) deletion or insertion, gel electrophoresis of the PCR products reveals the size difference. However, if the length polymorphism is caused by base substitution at a restriction site, PCR products must be digested with a restriction enzyme to reveal the RFLP. With the increasing number of 'universal' primers available in the literature, and depending on the amount of variation observed and the taxonomic level under examination, a researcher can target DNA regions that are either relatively conserved or rapidly evolving.

The potential power of RFLP markers to distinguish genetic variation is relatively low compared to more recently developed markers. The co-dominate nature of RFLP markers is its major strength, so and it remains unaffected by the

environment; any source of DNA can be used for the analysis because the size difference is often massive, and scoring is relatively easy. Also, sequence information is required for PCR analysis, making it difficult and time-consuming to develop markers lacking known molecular information.

2.7.4. Randomly Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) analysis is a new technology of molecular marking. RAPD procedure first developed in 1990 (Welsh and McClelland,1990; Williams *et al*., 1990) using PCR to randomly amplify anonymous segments of nuclear DNA with an identical pair of primers 8–10 bp in length and demonstrated the utility of single short oligonucleotide primers of arbitrary sequence for the amplification of DNA segments distributed randomly throughout the genome. Because the primers are short and relatively low annealing temperatures (often 36–40^0C) are used, the likelihood of amplifying multiple products is excellent, with each product (presumably) representing a different locus. RAPD markers are inherited as Mendelian markers dominantly and scored as present/absent. Homozygotes and heterozygotes produce an RAPD band, and though band intensity may differ, variations in PCR efficiency make scoring of band intensities difficult. As a result, distinguishing homozygous dominant from heterozygous individuals is not generally possible. Also, it is difficult to determine whether bands represent different loci or alternative alleles of a single locus so that the number of loci understudy assessed erroneously. RAPDs have all the advantages of a PCR-based marker. The added benefit is that primers are commercially available and do not require prior knowledge of the target DNA sequence or gene organization. Other advantages of RAPDs involve no blotting or hybridization steps, a large number of loci and individuals screened with ease. Hence, it is quick, simple, and efficient. RAPD markers used for species identification in fishes and mollusks, analysis of population structure in black tiger shrimp and marine algae, analysis of the genetic impact of environmental stressors, and genetic diversity analysis. However, they are no longer markers of choice as they lack reproducibility, co-migration, and robustness. Nearly all RAPD

markers are dominant; mismatches between primer and template may result in the total absence of PCR product, highly sensitive and complicated procedure, and cannot compare across labs.

2.7.5. Amplified Fragment Length Polymorphism (AFLP)

AFLP is a PCR-based, multi-locus fingerprinting technique that has procured attention among animal geneticists. AFLP has the potential to combine the strength and overcome the weakness of the RFLP and RAPD. Like RAPD, the molecular basis of AFLP polymorphism includes indels between the restriction sites and base substitutions at restriction sites, identical to RAPD. It also contains base substitution at PCR primer binding sites (Liu & Cordes, 2004). The adapter of the known sequence added to DNA fragments generated by digestion of whole-genome sequence, which is the remarkable feature of the technique that allows for the subsequent PCR amplification of a subset of the total pieces for ease of separation by gel electrophoresis. AFLP shows some characteristic similarity to RFLP and RAPD. Many loci are analyzed simultaneously for identifying genetic variation. Whereas, in RFLP, only one locus is analyzed at a time to identify genetic variation. Like RAPD, the AFLP marker is also inherited as dominant markers, and it does not require any prior molecular information. Thus it is a suitable marker for any species, including less well-studied fish species. Bands of AFLP are considered to be bi-allelic similar to RAPD. Therefore, it posses relatively low PIC scores, but the larger number of loci that can simultaneously be significantly scored increases their utility. Botanical and mycological literature described the crucial implementation of AFLP data for reconstructing phylogeny among closely related organisms. There has been the rapid application of this data in animal systems for which sequence-based studies have been inconclusive (Sullivan *et al*., 2003).

2.7.6. Single Nucleotide Polymorphism (SNP)

Point mutations like single nucleotide substitutions (transition/transversion) or single nucleotide insertions/deletions, give rise to SNPs. It is gaining attention in molecular marker development since they show the most abundant polymorphism in any organism's genome (coding and non-coding regions) flexible to automation, and disclose hidden polymorphism not detected with other markers and method. Theoretically, an SNP within a locus can produce as many as two alleles, each containing two possible base pairs at the SNP site. Therefore, SNPs are regarded as bi-allelic. SNP markers are inherited as co-dominant markers. Several approaches have been used for SNP discovery, including SSCP analysis (Hecker *et al*., 1999), heteroduplex analysis (Sorrentino *et al*., 1992), and direct DNA sequencing. DNA sequencing has been the most accurate and most used approach for SNP discovery (Liu & Cordes, 2004). SNPs have limitations such as difficulty in resolving mixed profile, development of SNP marker is labor-oriented, and some SNP assay techniques are costly.

2.7.7. Expressed Sequence Tags (ESTs)

ESTs are single sequences generated from random sequencing of cDNA clones (Adams *et al.,* 1991). ESTs are used to identify genes and analyze their expression by means of expression profiling (Franco *et al*., 1995; Azam *et al*., 1996; Lee *et al*., 2000). It helps in rapid and valuable analysis of genes expressed in specific tissue types, under specific physiological conditions, or during distinct developmental stages. ESTs offer the development of cDNA microarrays that allow the analysis of differentially expressed genes to be determined in a systematic way in addition to their tremendous value in genome mapping (Wang *et al.,* 1994). ESTs have their application for the identification of genes and analyzing. During specific developmental stages or under specific physiological conditions, ESTs aids in the quick and valuable analysis of genes expressed in particular tissue types. For genome mapping, ESTs are most useful for linkage mapping and physical mapping

in animal genomics such as cattle and swine, where radiation hybrid panels are available for mapping non-polymorphic DNA markers (Cox *et al*., 1990)**.** A radiation panel is composed of hybrid cells lines, with each hybrid cell containing small fragments of irradiated chromosomes of the species of interest. Generally, chromosomes are broken into small fragments by means of radiation to the cells from species of interest. It is impossible for radiated cells to survive by themselves.

Nevertheless, the radiated cells can be fused with recipient cells to form hybrid cells retaining a small part of the radiated chromosome. When chromosomal breakpoints are depicted within many mixed cell lines, it allows linkage and physical mapping of marker and genes. Although ESTs are popular in mammalian genome mapping, radiation hybrid panels are still not available for aquaculture species. Since physical mapping using BAC libraries provides even higher resolution and the fact that BAC libraries are already available from several aquaculture species. Thus, ESTs are convenient for mapping in aquaculture species only if polymorphic ESTs are identified (Liu *et al*., 1999). The value of EST resources and applications of bioinformatics in aquaculture genetics/genomics is inevitable, and it is expected that various ESTs databases will serve as a wealthy source of genomic information not only for aquaculture geneticists but also for aquaculture physiologists, immunologist and biotechnologists (Liu & Cordes, 2004).

2.7.8. Microsatellites

Microsatellites, or simple sequence repeats (SSRs), represent a unique type of tandemly repeated genomic sequences, which are abundantly distributed across the genomes and demonstrate a high level of allele polymorphism. They are the co-dominant markers of relatively small size, which is easily amplified with the polymerase chain reaction. These features form the foundation for their successful application in a wide range of fundamental and applied biology and medicine fields, including forensics, molecular epidemiology, parasitology, population and conservation genetics, genetic mapping, and genetic dissection of complex traits. Microsatellites are tandem repeats of two to six nucleotides and are hypervariable. It

is broadly classified as mono (e.g., T), di (e.g., CG), tri (e.g., CCG), or tetra (e.g., ATCG) nucleotide based on the no. of base pairs in a repeat unit (Krishnan, 2013). Higher-order SSR classes (tri-, tetra-, Penta- and hexanucleotides) are about 1.5 fold less common in the genomic DNA of vertebrates than dinucleotides (Toth *et al*., 2000). Dinucleotides are the most abundant among the microsatellites, which is classified based on base pairs. It is 2.3 folds more frequent. There are four types of microsatellite loci. Perfect loci have the same motif repeated tandemly; imperfect loci have the identical tandem repeat sequences interrupted by intervening sequences; compound loci contain more than one adjacent repeat motifs, and complex loci have more than one kind of repeat motifs separated by intermediary sequences. Microsatellites are widely distributed throughout the genome, especially in the euchromatin of eukaryotes, and coding and non-coding nuclear and organellar DNA (Maria Lucia, Carneiro Vieira *et al.,* 2016). They are also present in smaller bacterial genomes (Gur-Arie *et al*., 2000). The presence of Microsatellites in eukaryotic genomes has been known since the 1970s (Bruford *et al*., 1996, Hamada *et al*., 1982), demonstrated a large number and wide occurrence of these sequences from yeast to vertebrates. In fisheries biology, these markers are widely used to assess the following: effective population size of stocks (Reilly et al., 1999), stock identification (Shaklee and Bentzen 1998), levels of inbreeding (Tessier *et a*l., 1997), population structure, and gene flow (DeLeon *et al*., 1997; Arnegard *et al.,* 1999), parentage (Knight *et al.,* 1998), and quantitative traits (Jackson *et al.,* 1998).

2.7.9. Evolution of Microsatellite

Chistiakov *et al*., 2006 gave two hypotheses to understand the evolution of microsatellite. First, the theory suggests that larger genomes should have proportionately more microsatellite loci i.e., there is a positive relationship between the number of microsatellite loci and genome size and a negative relationship between microsatellite dispersion, the average number of DNA bases separating adjacent microsatellite loci and genome size. The second hypothesis suggests that there will be class-specific differences in the frequency of microsatellite loci

attributable to variation in the abundance of microsatellite precursors. Mutation rates varied considerably among microsatellite loci, and slipped strand mispairing is considered to be a pre-dominant mutational mechanism by which microsatellites mutate and form new alleles (Schiotterer and Tautz 1992., Strand *et al.,* 1993). The potential size expansion of di- or tetra-nucleotide microsatellites in untranslated regions (UTRs), introns, and coding areas could disrupt the native protein and formation of new genes with frame-shift mutations (Liu *et al*., 1999). The critical feature of SSRs as molecular markers is their hyper mutability and hence, their hypervariability in species and populations. The microsatellite mutation rate is estimated as 10^{-2}-10^{-6}per generation (Ellegren, 2000), several orders of magnitude greater than that of regular non-repetitive DNA (Li *et al*., 2002). Analysis of $(AC)_n$ microsatellites in five vertebrate classes (mammals, birds, reptiles, amphibians, and fishes) showed that length is a significant factor influencing the mutation rate. A directional mutation towards an increase in microsatellite length was also observed (Neff and Gross, 2001). Different models have come to explain and predict the mutation process that affects microsatellite evolution. The infinite allele model (IAM) assumes that the mutation microsatellite has the same probability of gaining or contracting one repeat unit. Thus, this model also takes into account back mutations. The generalized stepwise model (GSM) or two-phase model (TPM) is an extension of the SMM and considers the probability for a microsatellite mutation to involve more than one unit. According to the K-allele model (KAM), there are K allelic states and equal chances of mutating towards any of the other (K-1) alleles (AAnmarkrud *et al.,* 2008).

2.8. Advantages and Disadvantages of Microsatellites

Microsatellites have become a marker of choice. They are co-dominant, inherited in Mendelian fashion, and tandem arrays of concise repeating motifs 2-8 DNA bases can be repeated up to approximately 100 times a locus. They are among the fastest evolving genetic markers. They are highly polymorphic. At the genomic level, microsatellites tend to evenly disseminate on the entire chromosome i,e, both within gene coding part introns (Lie *et al.,* 2001) and non-coding sequences. Due to

the small-sized microsatellite loci, it is easy to amply microsatellite using PCR and subsequently analyze the amplified products on either manual sequencing gels or automated sequencing (Hakim Mudasir Maqsood, Syed Mudasir Ahmad, 2017). Only small amounts of tissues are required for typing microsatellites. These markers can be assayed using non-lethal fin clips and archived scale samples, facilitating retrospective analyses and depleted populations (McConnell *et al.,* 1995). A significant drawback of microsatellites is null alleles (O" Reilly and Wright, 1995; Pemberton *et al.,* 1995; Jarne and Lagoda, 1996). Moreover, it is expensive to develop species-specific primers for alleles and is time-consuming to identify adequate marker sets.

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1. Collection of Samples

Zebrafish samples for the present work were collected three different sites of Ziro, Arunachal Pradesh (26°50 N - 98° 21 N latitude and 92°40 E - 94°21 E longitude). Fish samples of 20 fish from each sites were collected from this three sites (1) A Fish pond adjacent to the paddy field in Hari village, Ziro, Arunachal Pradesh (2) A small canals in the paddy field in Hari village, Ziro, Arunachal Pradesh (3) A small stream connecting paddy field in Hija village, Ziro, Arunachal Pradesh. Distance between these two sampling sites is only 9 km.

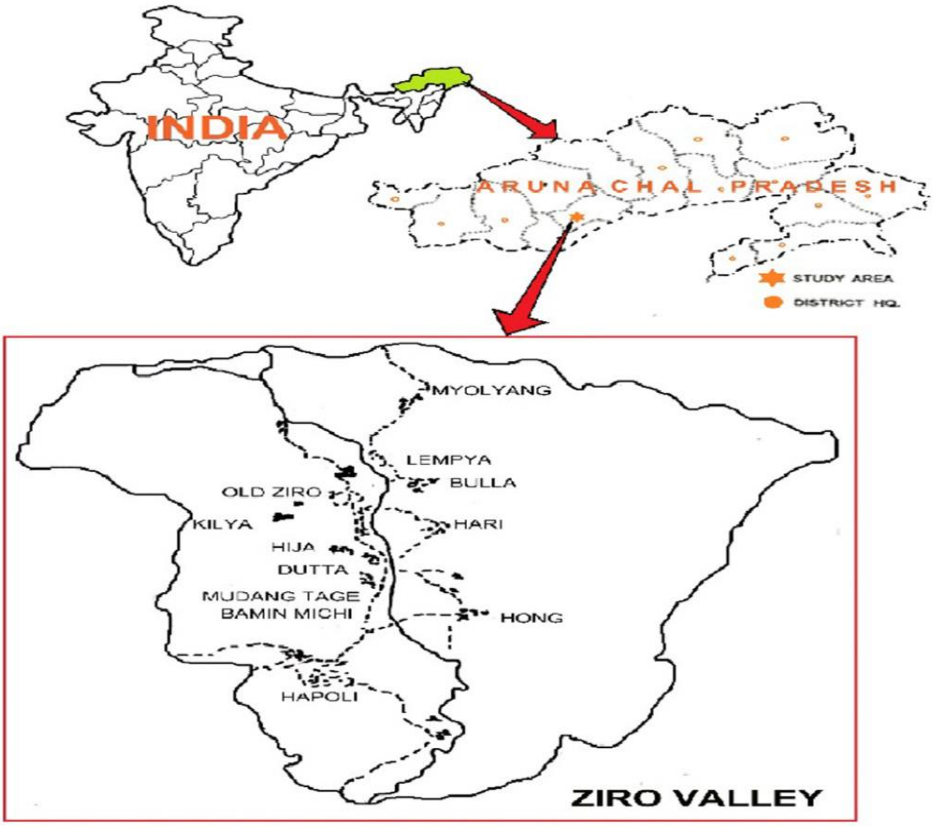

Fig.1. Map of Ziro in Arunachal Pradesh, India

Fig.2.Sampling site 1. A Fish pond adjacent to paddy field in Hari Village, Ziro, Arunachal Pradesh.

Fig.3.Sampling site 2. A small canal in the paddy field in Hari village, Ziro, Arunachal Pradesh.

Fig. 4.Sampling site 3. A small stream connecting paddy field in Hija Village, Ziro, Arunachal Pradesh.

3.2. Anaesthetizing of Fish Samples

Zebrafish samples were anesthetized by MS-222 solution before recording the sex. A stock solution of 1.5g/L was prepared and was buffered by adding sodium bicarbonate to system water and homogenized properly until pH of 7.2-7.4 was recorded. Zebrafish were placed in the anaesthetic water bath immediately after anaesthetic solution was homogenized. Time count started and various behaviour of zebrafish were noted such as slight loss of equilibrium when the fish began to show ventral to dorsal decubitus position, until the fish no longer moved, the time of anaesthetic induction was measured i,e, from the immersion point till the fish showed permanent imbalance in the bottom of the beaker in the dorsal decubitus position, the response of the soft stimuli and painful stimuli were tested every 10 sec. A plastic pipette was used to touch the lateral side of the fish inorder to assess the response to a soft stimulus and a forcep was used to evaluate a tail pinch by gently pressing the caudal fin and opercular movements were counted during 1 min to assess the respiratory rate after loss of equilibrium and loss of response to a soft touch.

3.3. Image collection

Photographs of every fish were captured to record standard length and body depth. The photographs were captured using Samsung SM-M307F mobile phone rear camera (48 megapixel primary sensor with an f2.2 aperture) in JPEG format with a resolution of 3264×1836 pixels.The standard length and body depth measurements were retrieved later by software, ImageJ (version 1.51) developed by Wayne Rasband of National Institute of Health, USA.

3.4. Recording of Sex

Sex of the fish was recorded using traditional method i,e, by visual observation of body shape, male had slender body shape and had a golden colour in the ventral region where as female was rounded and silvery and the presences of genital papilla in females. For fish whose genital papilla was poorly developed with intermediate

body shape, gentle squeezing was done and was checked for eggs or sperm. In absences of either, individual method of culling was adopted and dissected under a stereo microscope and their gonads analysed.

3.4.1. Steps involved in fish dissection for gonad analysing

Anaesthetized fish is placed on the dissecting mat, through the fleshy part of tail and ventral part of the eye socked the fish is pinned to the dissecting mat. Then, the skin and underlying muscle is cut along the belly from the anal fin to the operculum. The operculum, pectoral fin including pectoral girdle is removed and muscle is cut from above the exposed gill posterior along the side of the fish and then down to the anal fin using a forceps and a surgical scissor. Many of the internal organs such as heart, kidney, spleen, liver, swim bladder and gall bladder are now visible, after the skin and underlying muscle from the side of the fish is carefully removed a testis is observed in case the fish is male and a bi-lobed ovary suspended in the body cavity is seen in female. The testis are long, white paired organs that remains attached to the dorsal body wall. Both testis and ovary is removed and placed in a dish of PBS and examined under reflected light. In testis, seminiferous tubules were observed which contained cysts with various stages of developing germ cells from spermatogonia to spermatids and an oocytes were observed in ovary which when teased apart using a fine needles, showed different stages of oocytes.

3.5. Site of Experiment

The research work was carried out in Fish Genetics and Biotechnology Division, ICAR-Central Institute of Fisheries Education, Mumbai.

3.6. Retrieving of information from images using ImageJ tool

The morphometric traits of 60 fish, comprising equally from each sites were recorded for the study. The photographs of all fish were recorded to get the values of all parameters. The photographs were captured using using Samsung SM-M307F mobile phone rear camera (48 megapixel primary sensor with an f2.2 aperture) in JPEG format with a resolution of 3264×1836 pixels. Twelve morphometric traits *viz*., **TL** – Total Length, **SL** - Standard length, **ED** – Eye diameter, **POL** – Pre-Opercular length, **UJVO**- Distance between the upper jaw tip and ventral tip of operculum, **AFBL** – Anal fin base length, **DFBL** - Dorsal fin base length, **CFL** – Caudal fin length, **MBD** – Maximum body depth, **CPD** – Caudal peduncle depth, **AFL** – Anal fin length, **DFL** – Dorsal fin length (Plate 5 and Table 1), were further retrieved using the software, ImageJ (version 1.51).

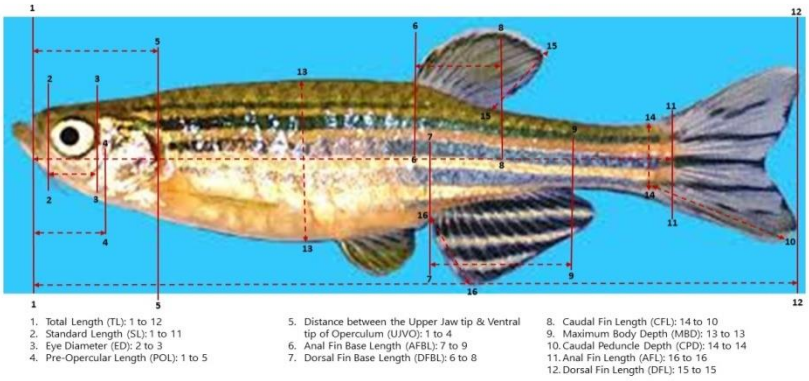

Fig.5.Morphometric traits of zebrafish retrived from imageJ software

Table 1: Morphometric characters used for the present study

Table.1. Morphometric traits used for present study

Sl No.	Morphometric traits	Acronyms	Description
1	Total length	TL	Length of the fish from anterior-most tip of the snout to the posterior-most tip of the longest lobe of the caudal fin. In figure 1, it is the linear distance between 1stand 11th line
2	Standard length	SL	Distance between the snout tip and the base of the caudal fin rays. In figure 1, it is the linear distance between 1st and 9th line.
3	Eye diameter	ED	The diameter of the eye orbit along the body axis. In figure 1, it is the linear distance between 2nd and 3rd line.
4	Pre – opercular length	POL	It is the distance from snout to an eye.
5	Distance between the upper jaw tip and ventral tip of operculum	UJVO	It is the distance measured from the tip of the upper jaw to the ventral tip of the operculum.
6	Anal fin base length	AFBL	It is the minimum linear distance from the origin of first anal fin ray to the origin of the last anal fin ray. In figure1,it is the minimum linear distance between 7thand8th line
7	Dorsal fin base length	DFBL	It is the minimum linear distance of the dorsal fin from the first origin of the fin to the insertion point of the fin. In figure 1, it is the linear distance between 5thand to 8th line.
8	Caudal fin length	CFL	It is the length of the longest fin ray of the caudal fin
9	Maximum body depth	MBD	It is the minimum linear distance between the opposite ends of the deepest part of the body.
10	Caudal peduncle depth	CPD	The distance from the anterior end of caudal peduncle to the ventral surface of the caudal peduncle at deepest part.
11	Anal fin length	AFL	It is the length of the longest fin ray of the anal fin
12	Dorsal fin length	DFL	Length of the longest fin ray of the dorsal fin

3.7. Sample Preservation

After taking morphometric readings of the zebrafish samples. Later, the samples were preserved in absolute alcohol. The alcohol was drained and replaced with fresh absolute alcohol after 15- 20 days. The samples were stored at -20^{o}C until further use. DNA was isolated from all the individuals by Phenol-Chloroform method and stored at -20^{0}C until further usage.

3.8. Chemicals and consumables

The chemicals were procured from Qualigens, SRL, India and Thermo Scientific, USA, except otherwise specified. DNA primers were from Xcelris, India; DNA size standards from Thermo Scientific, USA; PCR reagents were from SRL, India and Thermo Scientific, USA; Gel Extraction kit and Plasmid Extraction kit from Qiagen, USA. Glassware for the work was acquired from Borosil, India and plasticware from Tarsons Pvt. Ltd., India. Solutions were prepared in autoclaved demineralised water (DMW, MilliQ). All solutions, plastic ware and glassware except heat labile components were sterilized by autoclaving at 15lb for 20 min. Heat labile solutions were filter sterilized using 0.22 micron cellulose acetate disposable syringe filters (Osmonics, USA). The reagents used in this work are detailed in Appendix I& II.

3.9. Equipment and Laboratory-ware

Thermocyclers (Biorad, USA and Applied Biosystems, USA), Nanodrop spectrophotometer(USA), refrigerated centrifuge (Heraeus, Germany), horizontal gel electrophoresis apparatus (Thermo Scientific, USA), gel documentation system (BioRad, USA), electronic balance (OHAUS, USA), table-top centrifuge 5430 (Eppendorf, Germany), microwave oven (Kenstar,India), autoclave and micropipettes (Eppendorf, Germany) were the equipment used in this work.

3.10. Genomic DNA Isolation

Approximately 100 mg muscle tissue was taken in a fresh 2 ml microfuge tube using sterile scissors and forceps and cut into small pieces. To this 500µl TEN lysis buffer, 50 µl SDS (10%) and 10µl proteinase K (1mg/ml) were added and the tube was incubated at 55°C in a water bath for 18-20 h. After lysis equal volume of Tris-saturated phenol (pH 8.0) was added and mixed gently for proper emulsification and lysate was centrifuged at 10,000 rpm for 10 min. The top aqueous layer was collected carefully into a fresh 2 ml microfuge tube and extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 v/v). The supernatant was once again extracted with equal volume of chloroform: isoamyl alcohol (24:1 v/v).After centrifugation the top aqueous layer containing DNA was transferred to a fresh microfuge tube. To this one-tenth volume of 3M sodium acetate, pH 5.2 was added and DNA was precipitated with 0.6 volumes of isopropanol. The tube was centrifuged at 10000 rpm for 10 min at 4°C to pellet the DNA and supernatant was discarded. The DNA pellet was washed with 70% chilled ethyl alcohol and centrifuged at 10000 rpm for 5 min to remove excess salt. The DNA pellet was air dried and dissolved in an appropriate volume of TE buffer and 1 µl of RNase (Annexure I) was added and incubated at 37^0C for 1 hr to remove RNA. The DNA was kept at 4^0C for short term and at -20^{o}C for long term storage.

3.10.1. Quality Check and Quantification of DNA

The integrity of the isolated DNA was checked by agarose gel electrophoresis on 1% agarose gel. Appropriate amount of agarose (0.3 g) was added to 30 ml of 0.5 X TAE buffer and melted in a microwave oven till the solution became completely clear. Ethidium bromide (2 µl of 1% solution) was added to the agarose solution after cooling to about 60^{o}C and poured into the gel casting tray (7 x 10 cm) and allowed to solidify at room temperature. The comb was removed carefully after 30 min and the gel tray was placed in the gel electrophoresis tank

containing 0.5 X TAE buffer to a level just above gel. Appropriate volume of 0.5 µl of DNA was mixed with 6X gel loading dye and electrophoresis buffer and loaded into the well. The unit was connected to power supply and the gel was run at 80 V for 1 h. The gel was then taken out and documented in a gel documentation unit (BioRad, USA). Quantity was also checked in nanodrop spectrophotometer that also provided the 260/280 value that is a measure of DNA purity.

3.11. PCR Amplification of Microsatellite loci

Genomic DNA was amplified in sterile 0.2ml PCR tube using BIO-RAD 100 thermal cycler. The final amplification reactions were carried out in 25µl reaction mixture prepared as given below.

10x Buffer with $MgCl_2$	2.5 µl
Template (50-100ng)	1.0 µl
dNTP (200µM)	2.0 µl
Forward primer(10 pmol)	1.0 µl
Reverse primer(10 pmol)	1.0 µl
Taqpolymerase (1 U)	0.30 µl
DMW	To make up to 25 µl

The cycler was programmed for initial denaturation at 94ºCfor 5 min and 35 cycles of denaturation at 94ºC for 30s, primer specific touchdown annealing temperatures for 30s and 1 min at 72ºCfor extension. The final extension was set at 72ºC for 7 min followed by hold at 4ºC.

3.12. Polyacrylamide Gel Electrophoresis (PAGE)

Amplified products were separated by PAGE (polyacrylamide gel electrophoresis). As the expected amplicon sizes were in the range of 160-350bp,

10% resolving gel was used. The PAGE apparatus (Technosource, India) was assembled and sealed with 1% agarose before pouring the gel mixture. Spacers and combs of 1 mm thickness were used. A 30% stock solution of acrylamide and bisacrylamide was prepared as detailed in Appendix I and stored at 4ºC until use in a dark bottle. Gel mixture for 10% resolving gel was prepared. Ammonium persulphate solution was prepared fresh in DMW.

3.12.1. Reagents required

The mixture was poured between the plates, and the comb was inserted carefully so as not to introduce bubbles. The gel was allowed to polymerize for 30 min, and then the anodic and cathodic chambers were filled with a 1x TAE buffer. The comb was gently removed, and the samples were loaded. The samples included 15µl of the PCR product and3µl of 6X loading dye. The apparatus was connected to a power supply and run under constant voltage conditions at 80 V for 7-8 hours, allowing adequate time for allele separation. After the run, the apparatus was dismantled, and the gel was subjected to Ethidium bromide staining. The stained gels were visualized on the gel documentation system (BioRad, USA).

3.13. Microsatellite allele scoring

The size of the amplicons of microsatellite loci (alleles) were estimated using the DNA size ladder by MyImage Analysis software v2.0 (ThermoFisher Scientific, USA). Alleles were identified and the genotypes were assigned to each individual based on the number of alleles and their mobility pattern on the gel. Since microsatellites are codominant, an individual displaying single amplicon was considered as homozygote for that respective allele, whilst individual revealing two amplicons was attributed as heterozygote at that locus. An illustration of image analysis is given in figure 32-37.

3.14. Statistical analysis of data

The genetic parameters viz., allele frequencies, observed and expected heterozygosity values and genetic differentiation were estimated using GenAlex 6.4 (Peakall and Smouse, 1996) software. Polymorphic information content (PIC) values were estimated for all loci using Cervus v.3.0 (Marshall *et al*., 1998).

3.14.1. Descriptive statistics

Descriptive statistics viz., number of observations (N), minimum, maximum, mean, standard error, standard deviation and coefficient of variation (CV) were estimated on raw data by employing the Proc means procedure of SAS® 9.4 system (SAS Institute Inc., 2016, version 9.4).

3.14.2. Analysis of variance and estimation of least squares means

The significant sources of factors influencing the standard length and body weight were estimated by adopting fixed effect model. The data after correcting for normality were analyzed employing PROC GLM of SAS (SAS Institute Inc., 2016, version 9.4) for the Analysis of Variance (ANOVA). The model adopted for least square means was

$$\mathbf{Y_{ij} = \mu + gender_i + e_{ij}}$$

where, $\mathbf{Y_{ij}}$ is the j^{th} observation of the individual of i^{th} gender

$\boldsymbol{\mu}$ is the overall mean

$\mathbf{gender_i}$ is the fixed effects due to sex of the animal

$\mathbf{e_{ij}}$ is the random error associated with the $\mathbf{Y_{ij}}$. Random error is assumed to be i.i.d, N~ $(0,\sigma^2_e)$

Table 2:- Primer Information

Sl no	Primer ID	Primer	Product (bp)	Type of Repeat	Repeat	Ta (°C)
1.	Z20576	F: 5´-GTCTCAGGCAGCTGTCACATGATG-3´ R:3´-TTTCAAGGGCAATAGGGCTGAAC-5´	102	Tri	(ATC)12	66.3
2.	Z7141	F: 5´- AGGAGAGAGTGGAGTCTAATGACTGC-3´ R: 5´- ATATATGGCACATTCACAATACCTCG-3´	168	Di	(TG)37	64.1
3.	Z7156	F: 5´-TGTCCTCCTCAAACGTCTCCTG-3´ R: 3´-GCAGGTTTAGAGCACATCACAGC-5´	140	Di	(AC)13	66.0
4.	Z10215	F: 5´-TGATCAGGTGTCGTCCTCAGCGTG-3´ R: 3´-TGTTCTGCTGTTTAGACCATGTGAG-5´	186	Di	(TC)17	68.0
5.	Z20966	F: 5´-ACAACACTCAGCACCAGAACAGACC3´ R: 3´-GCCTTCAGCTTTAGTGGTTCAGTCCC-5´	168	Di	(CA)13	70.0
6.	Z1547	F: 5´-TCTTTCCAGATGGTTTCTGTTAAGGC-3´ R: 3´-AATGTGCTCCTTCGCGCTGATG-5´	208	Di	(GT)11	66.2

RESULTS

4. RESULTS

4.1. Morphometric Study

The Morphometric measurements of zebrafish for the study *viz*; **TL** – Total Length, **SL** - Standard length, **CPD-** Caudal peduncle length, **MBD** – Maximum Body Depth, **ED** – Eye diameter, **POL** – Pre- Opercular length, **UJVO** - Distance between upper jaw tip and ventral tip of operculum, **AFBL** - Anal fin base, **AFL** – Anal fin length, **CFL** – Caudal fin length, **DFBL** - Dorsal fin base length, **DFL** – Dorsal fin length are provided in the Fig. 1.

4.2. Descriptive Statistics

The descriptive statistics of the 12 morphometric parameters in zebrafish are provided in the Table 1. The records were collected on 61 adult zebrafish that comprised 22 males and 39 females. The overall mean value for the morphometric traits *viz*; total length, standard length, caudal peduncle length, maximum body depth, eye diameter, pre-opercular length, distance between upper jaw tip and ventral tip of operculum, anal fin base, anal fin length, caudal fin length, dorsal fin base length, dorsal fin length were 3.69 ± 0.06 cm, 2.98 ± 0.05 cm, 0.33 ± 0.01 cm, 0.78 ± 0.02 cm and 0.17 ± 0.00 cm respectively. The coefficient of variation (CV) of 6 morphometric parameters *viz*; total length, eye diameter, pre-orbital length, anal fin length, dorsal fin base length and dorsal fin length were found to be higher in males than females and for the rest of the traits females had a higher CV value viz., standard length, caudal peduncle depth, maximum body depth, distance between upper jaw tip and ventral tip of operculum, anal fin base length and caudal fin length. The within and between sex variation for the 12 morphometric parameters of zebrafish are provided as box plots in Fig. 2. to Fig. 8 respectively. The maximum and minimum total length recorded in zebrafish was 4.44 cm and 2.42 cm respectively. The maximum body depth recorded showed highest CV of 20.67.

4.3. Variation in morphometric parameters

The overall and sex wise least square mean values of all 12 morphometric parameters are provided in Table 2. 5.The LS means of female zebrafish for total length, standard length, caudal peduncle length, maximum body depth, pre-opercular length, anal fin base length and dorsal fin lengthwere 3.88 ± 0.06 cm, 3.14 ± 0.05 cm, 0.35 ± 0.01 cm, 0.86 ± 0.01 cm, 0.58 ± 0.01 cm, 0.51 ± 0.01 cm and 0.50 ± 0.01 cm respectively and were significantly higher when compared to male zebrafish. However, there was no significant difference between male and female zebrafish for the traits *viz*; eye diameter, distance between upper jaw tip and ventral tip of operculum, anal fin length, caudal fin length and dorsal fin base length.

The mean sum of square (MSS) values from analysis of variance for all the 12 morphometric parameters are represented in Table 3. The effect of sex was significant on total length, standard length and maximum body depth in zebrafish**.**

4.4. Analysis of morphometric traits as function of total length

The morphometric traits are analysed as function of total length using a simple linear regression equation Y = bX + a where 'Y' represents the value of morphometric trait that is expressed as function of total length and 'X' represents the value total length (TL), a and b are parameters; 'a' represents the X intercept and 'b' is the regression coefficient. The function along with the parameters for each morphometric trait is mentioned in Table 4. All the traits are positively correlated with total length. The traits *viz*; standard length and maximum body depth had highest correlation with total length and their values were> 0.9. Standard length had the highest regression coefficient (b) value of 0.82 mean while; eye diameter had the least value of 0.03. The goodness of fit is measured by R^2 and only two traits *viz*; standard length and maximum body depth had R^2 value > 0.7. The traits *viz*., dorsal fin base length, dorsal fin length, eye diameter, anal fin length, anal fin base length and distance between upper jaw tip and ventral tip of operculum had R^2 value < 0.5. The regression curves with 95% confidence limits for the traits *viz*; standard length, maximum body depth, caudal fin length, anal fin length, dorsal fin base length and dorsal fin length as a function of total length is given in figures 13 to 1

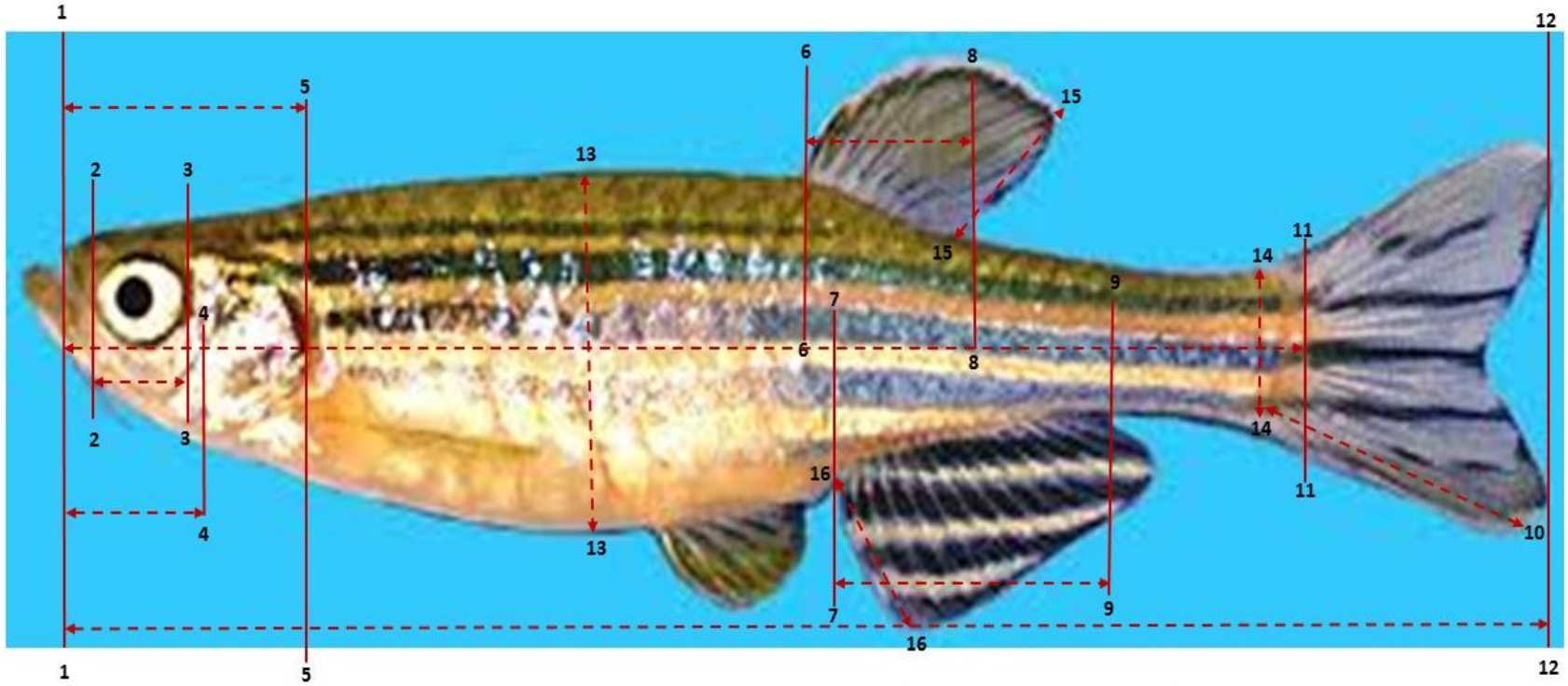

Fig.6. Morphometric measurements of *Zebrafish* for the study (TL – Total Length, SL - Standard length, CPD- Caudal peduncle length, MBD – Maximum Body Depth, ED – Eye diameter, POL – Pre- Opercular length, UJVO - Distance between upper jaw tip and ventral tip of operculum, AFBL - Anal fin base, AFL – Anal fin length, CFL – Caudal fin length, DFBL - Dorsal fin base length, DFL – Dorsal fin length.

Table 3: Descriptive statistics values of all morphometric parameters

Parameter	Source of variation	N	Means ± SE	CV	Minimum Value	Maximum Value
TL	Overall	61	3.69 ± 0.06	13.44	2.42	4.44
	Male	22	3.34±0.08	11.71	2.42	4.26
	female	39	3.88±0.07	11.28	2.49	4.44
SL	Overall	61	2.98 ± 0.05	13.80	1.99	3.62
	Male	22	2.69±0.07	11.35	2.02	3.38
	female	39	3.15±0.06	11.80	1.99	3.62
CPD	Overall	61	0.33 ± 0.01	17.24	0.19	0.45
	Male	22	0.30±0.01	15.83	0.20	0.39
	female	39	0.35±0.01	15.98	0.19	0.45
MBD	Overall	61	0.78 ± 0.02	20.67	0.46	1.06
	Male	22	0.64±0.02	15.15	0.46	0.83
	female	39	0.86±0.02	15.52	0.48	1.06
ED	Overall	61	0.17 ± 0.00	16.71	0.11	0.22
	Male	22	0.16±0.01	18.40	0.11	0.21
	female	39	0.18±.000	15.41	0.12	0.220
POL2	Overall	61	0.56 ± 0.01	14.29	0.40	0.72
	Male	22	0.51±0.02	14.46	0.40	0.68
	female	39	0.59±0.01	11.92	0.44	0.72
UJVO	Overall	61	0.50 ± 0.01	16.55	0.29	0.69
	Male	22	0.47±0.01	14.23	0.33	0.56
	female	39	0.52±0.01	16.52	0.29	0.69
AFBL	Overall	61	0.50 ± 0.01	14.39	0.31	0.69
	Male	22	0.46±0.01	11.15	0.34	0.57
	Female	39	0.52±0.01	14.32	0.31	0.69
AFL	Overall	61	0.48 ± 0.01	15.79	0.28	0.66
	Male	22	0.46±0.02	17.04	0.28	0.59
	female	39	0.49±0.01	14.70	0.33	0.66
CFL	Overall	61	0.75 ± 0.01	15.35	0.38	0.95
	Male	22	0.70±0.02	12.92	0.49	0.89
	female	39	0.77±0.02	15.39	0.38	0.95
DFBL	Overall	61	0.27 ± 0.01	18.59	0.15	0.43
	Male	22	0.27±0.01	20.81	0.15	0.35
	female	39	0.28±0.01	17.41	0.18	0.43
DFL	Overall	61	0.48± 0.01	15.18	0.32	0.62
	Male	22	0.44±0.01	14.25	0.32	0.56
	female	39	0.50±0.01	13.60	0.36	0.62

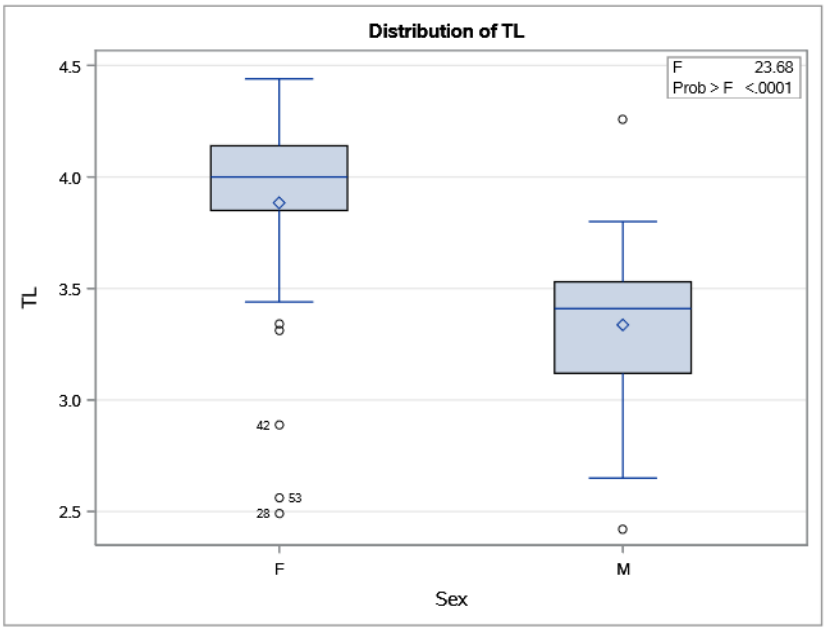

Fig.7.Box and Whisker plot showing sex wise variation of total length in centimeters

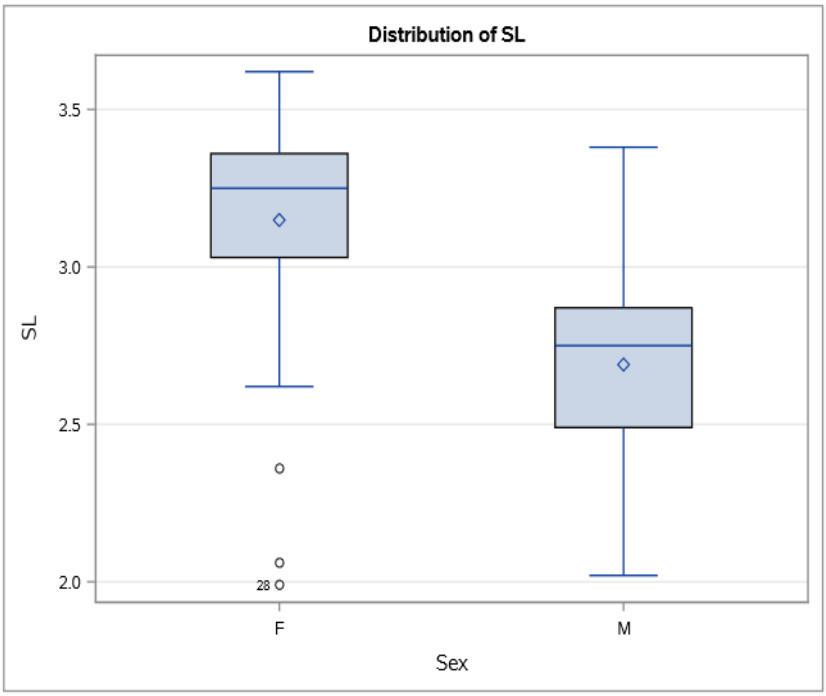

Fig.8.Box and Whisker plot showing sex wise variation of standard length in centimeters

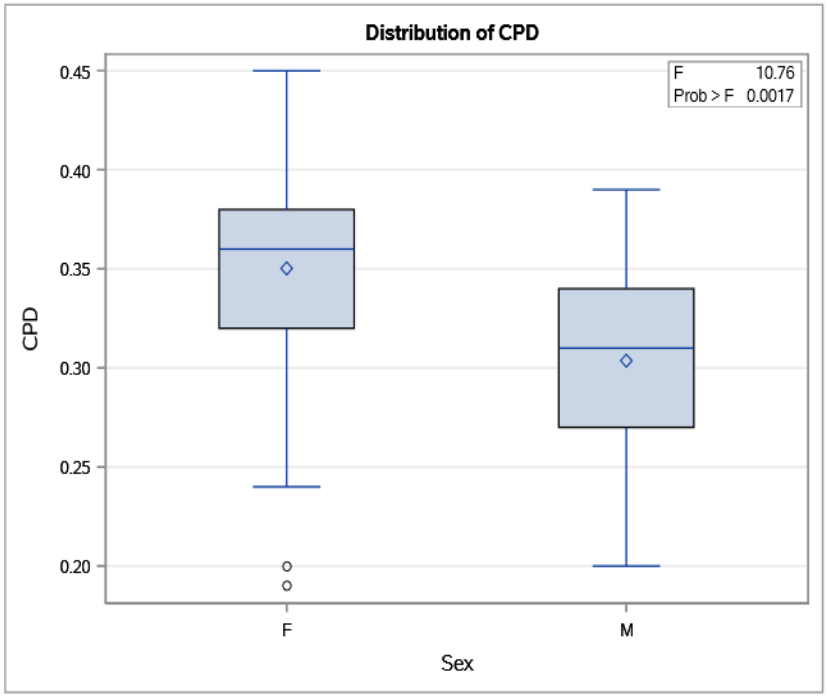

Fig.9..Box and Whisker plot showing sex wise variation of caudal peduncle depth in centimeters

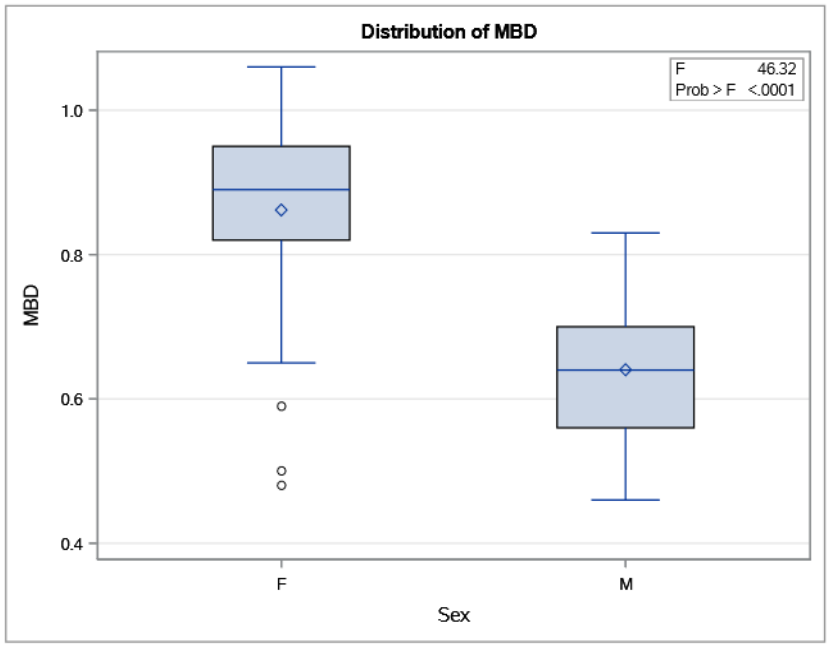

Fig.10.Box and Whisker plot showing sex wise variation of maximum body depth in centimeters

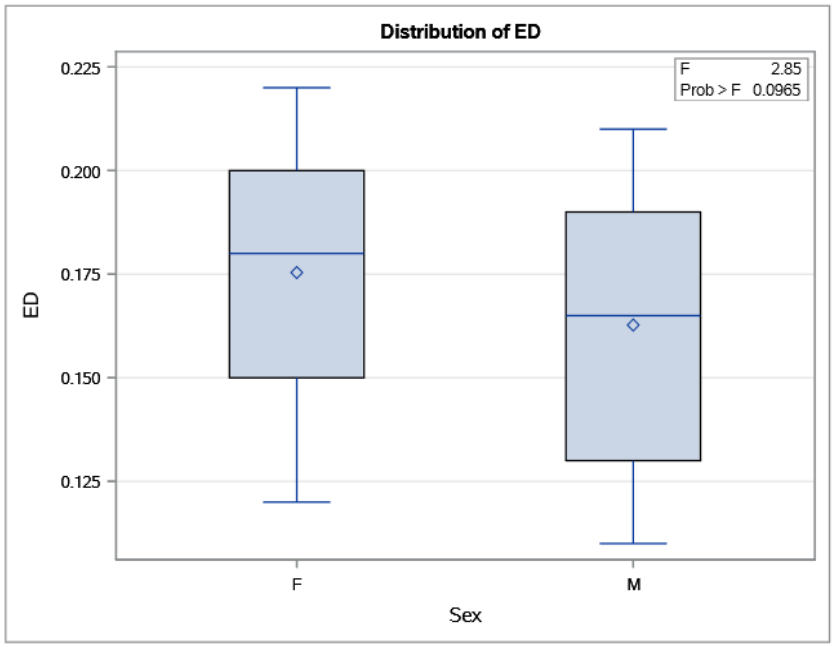

Fig.11.Box and Whisker plot showing sex wise variation of eye diameter in centimeters

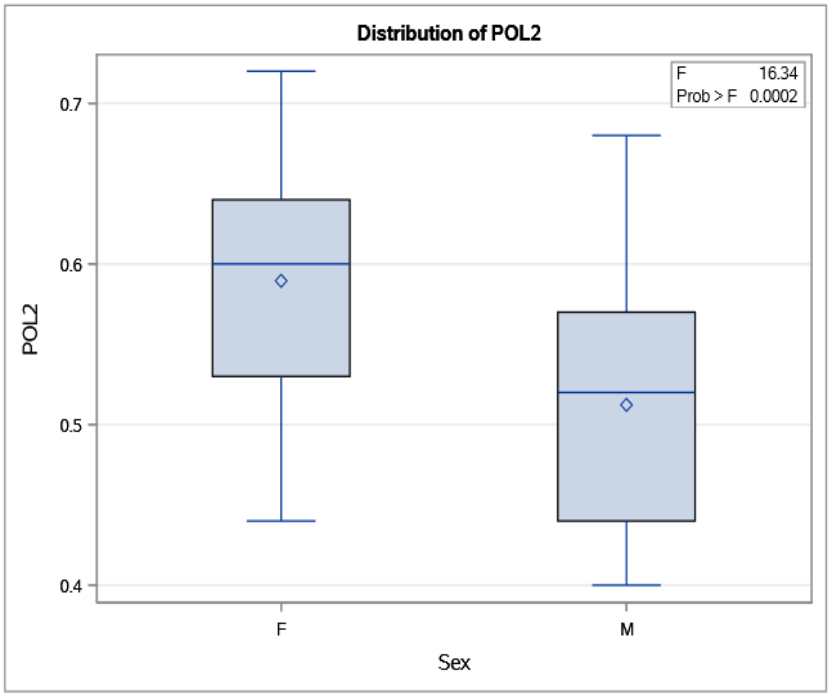

Fig.12.Box and Whisker plot showing sex wise variation of pre-opercular length in centimeters

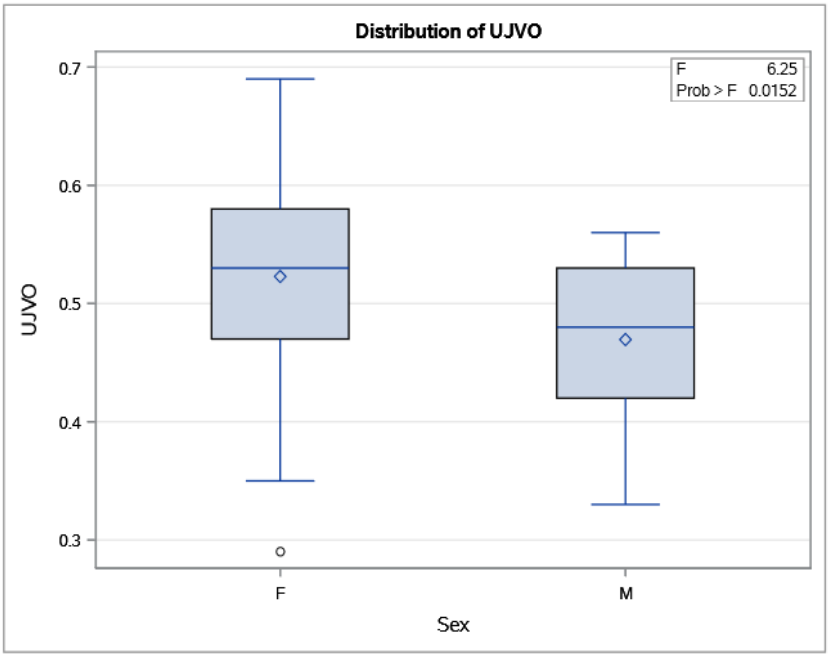

Fig.13.Box and Whisker plot showing sex wise variation of distance between upper jaw tip and ventral tip of operculum in centimeters

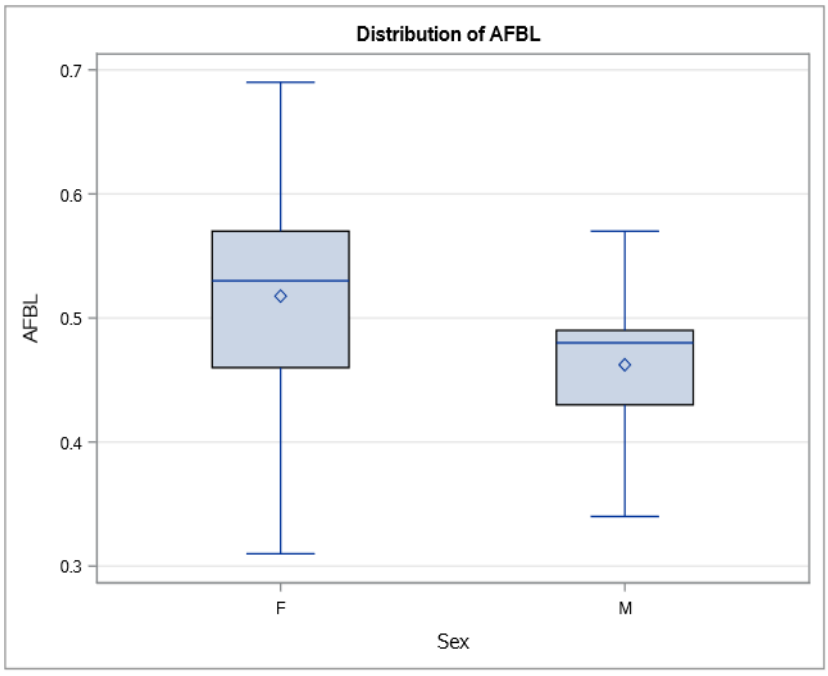

Fig.14.Box and Whisker plot showing sex wise variation of anal fin base length in centimeters

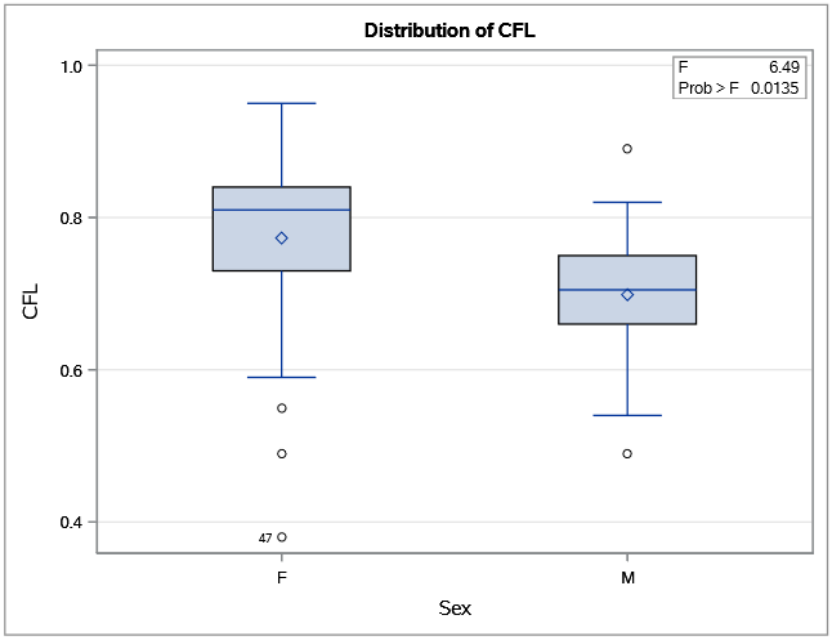

Fig.15.Box and Whisker plot showing sex wise variation of caudal fin length in centimeters

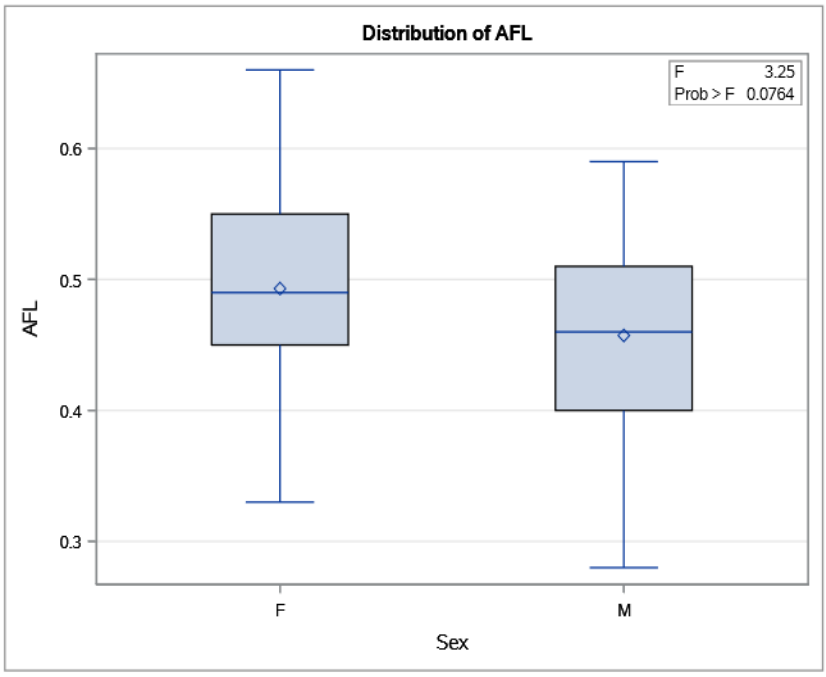

Fig.16.Box and Whisker plot showing sex wise variation of anal fin length in centimeters

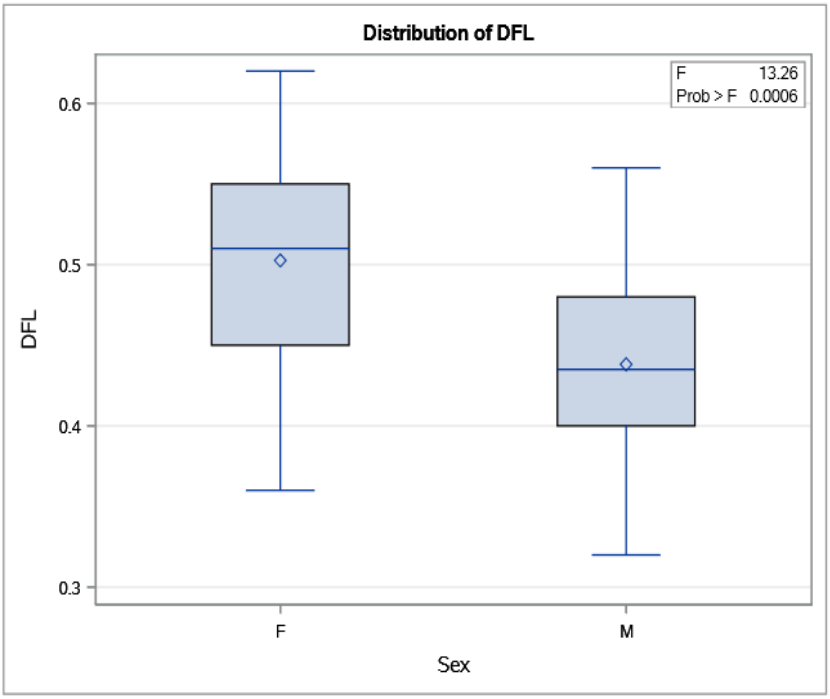

Fig. 17.Box and Whisker plot showing sex wise variation of dorsal fin length in centimeters

Table 4: Sex wise least square means (cm) for morphometric parameters

Sl No	Parameters	Sex		Overall
		Female	Male	
1	Total length	3.88 ± 0.06[a]	3.33 ± 0.08[b]	3.69 ± 0.06
2	Standard Length	3.14 ± 0.05[a]	2.69 ± 0.07[b]	2.98 ± 0.05
3	Caudal Peduncle Length	0.35 ± 0.01[a]	0.30 ± 0.01[b]	0.33 ± 0.01
4	Maximum Body Depth	0.86 ± 0.01[a]	0.64 ± 0.02[b]	0.78 ± 0.02
5	Eye Diameter	0.17 ± 0.004[a]	0.16± 0.005[a]	0.17 ± 0.00
6	Pre-Opercular Length	0.58 ± 0.01[a]	0.51 ± 0.01[b]	0.56 ±0.01
7	Distance between Upper Jaw tip & Ventral tip of Operculum	0.52 ± 0.01[a]	0.46 ± 0.01[a]	0.50 ±0.01
8	Anal Fin Base Length	0.51 ± 0.01[a]	0.46 ± 0.01[b]	0.50±0.01
9	Anal Fin Length	0.49 ± 0.01[a]	0.45 ± 0.01[a]	0.48 ±0.01
10	Caudal Fin Length	0.77 ± 0.01[a]	0.69 ± 0.02[a]	0.75±0.01
11	Dorsal Fin Base Length	0.27 ± 0.01[a]	0.26 ± 0.01[a]	0.27 ±0.01
12	Dorsal Fin Length	0.50 ± 0.01[a]	0.43 ± 0.01[b]	0.48±0.01

Table 5: Mean sum of squares from analysis of variance for different morphometric parameters

Source of Variation	DF	Morphometric Parameters											
		TL	SL	CPD	MBD	ED	POL	UJVO	AFBL	AFL	CFL	DFBL	DFL
Sex	1	4.21*	2.96*	0.03^{NS}	0.68*	0.002^{NS}	0.08^{NS}	0.03^{NS}	0.043^{NS}	0.01	0.07^{NS}	0.001^{NS}	0.05^{NS}
EMS		0.17	0.12	0.002	0.01	0.0007	0.005	0.006	0.004	0.005	0.01	0.002	0.004
Error DF		59	59	59	59	59	59	59	59	59	59	59	59
R^2(%)		28.6	29.1	15.4	43.9	4.6	21.6	9.5	14.0	5.2	9.9	1.2	18.3

* $p<0.01$, NS- not significant ($p>0.01$)

Table 6: Regression coefficient (b) for various morphometric parameters as function of total length (TL)

Parameters	a	b	r	R^2	Y = bX + a
Standard Length	-0.05	0.82	0.98	0.98	Y= 0.82TL -0.05
Caudal Peduncle Depth	-0.005	0.091	0.79	0.62	Y= 0.091TL - 0.005
Eye Diameter	0.04	0.03	0.56	0.32	Y= 0.03TL +0.04
Pre-Opercular Length	0.12	0.11	0.73	0.54	Y= 0.11TL +0.12
Distance between upper Jaw tip and Ventral tip of Operculum	0.09	0.11	0.66	0.44	Y=0.11TL +0.09
Anal Fin Base Length	0.14	0.09	0.66	0.44	Y=0.09TL +0.14
Anal Fin Length	0.09	0.10	0.67	0.45	Y=0.10TL +0.09
Caudal Fin Length	0.11	0.17	0.73	0.54	Y=0.17TL +0.11
Dorsal Fin Base Length	0.11	0.04	0.42	0.18	Y=0.04TL +0.11
Dorsal Fin Length	0.11	0.10	0.67	0.46	Y=0.10TL +0.11
Maximum Body Depth	-0.35	0.30	0.94	0.89	Y= 0.30TL -0.35

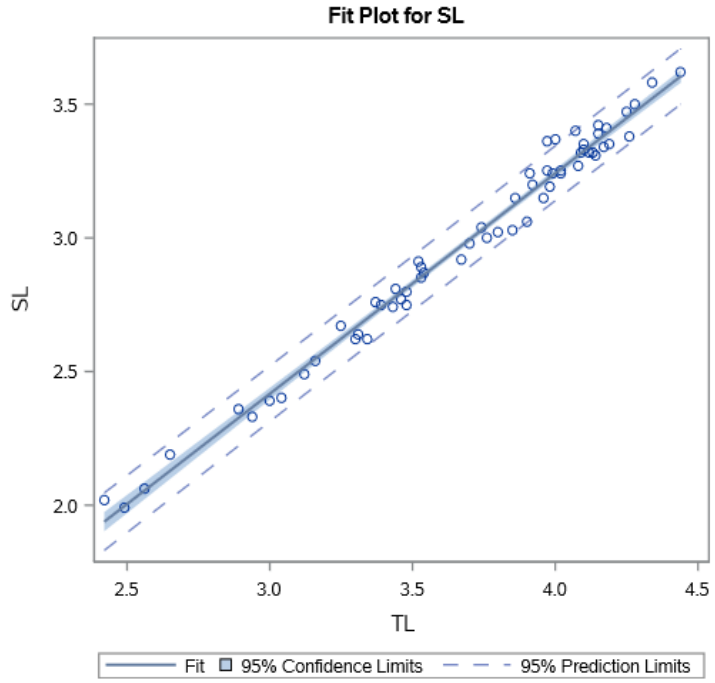

Fig. 18.Regression curve of standard length on total length

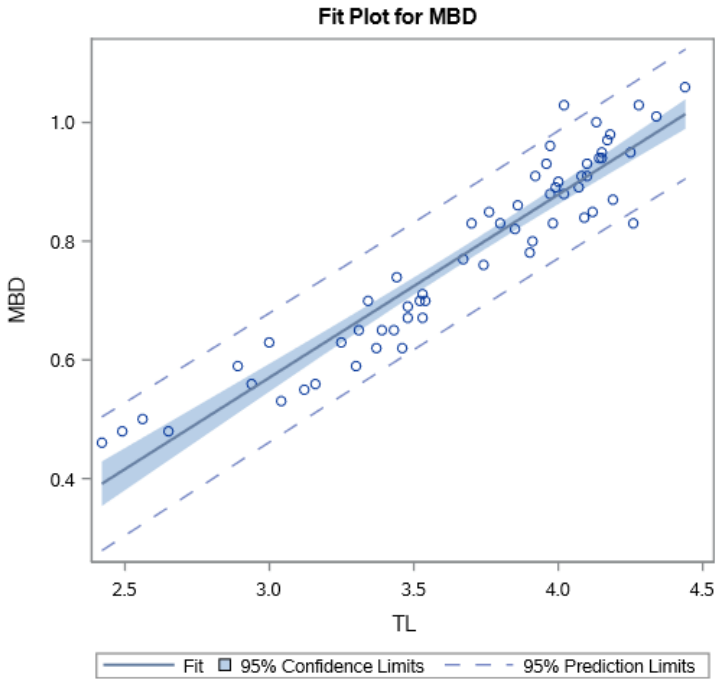

Fig. 19.Regression curve of maximum body depth on total length

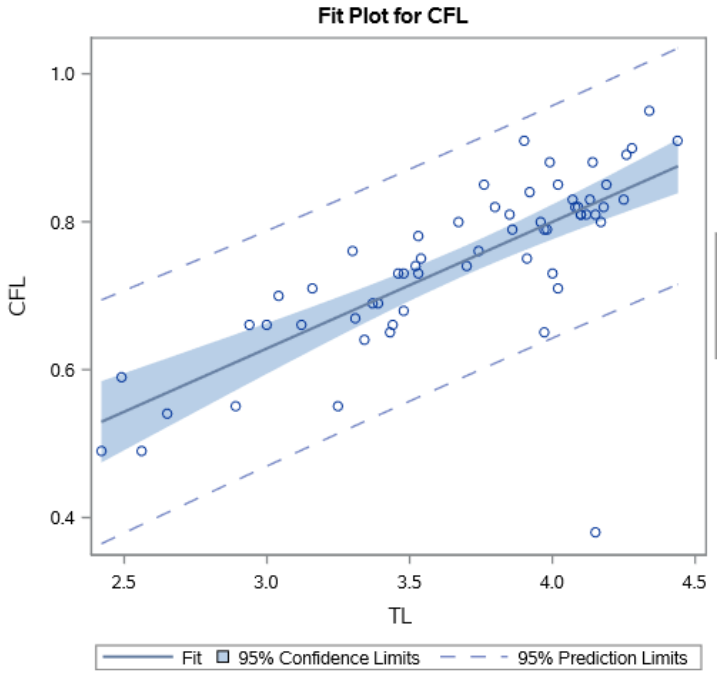

Fig. 20.Regression curve of caudal fin length on total length

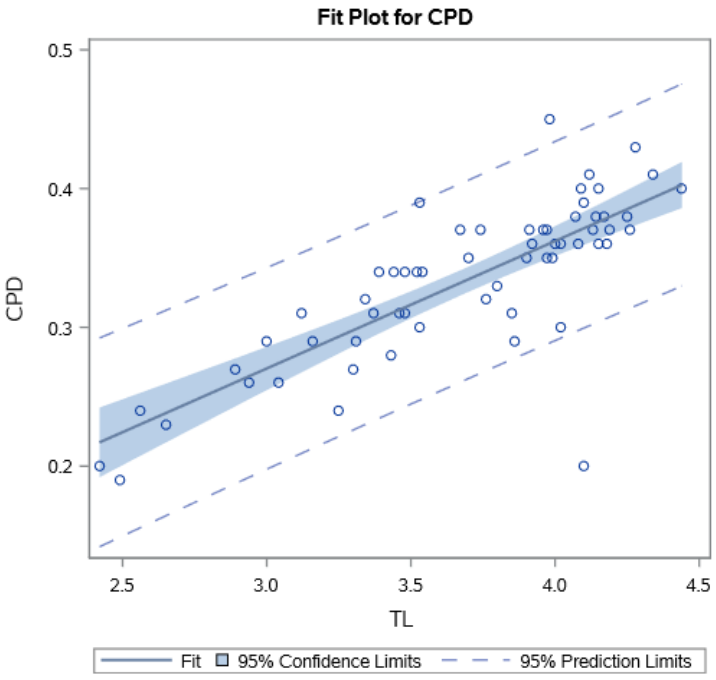

Fig.21.Regression curve of caudal peduncle depth on total length

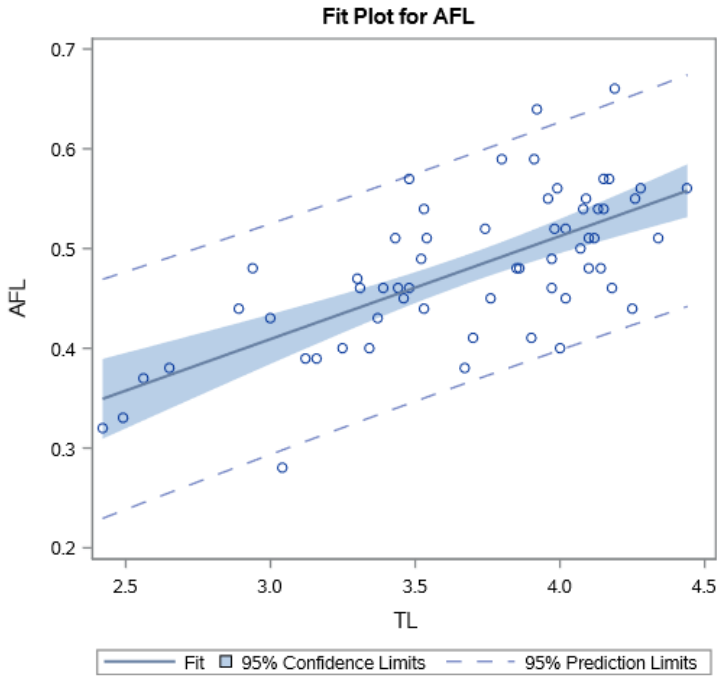

Fig.22.Regression curve of anal fin length on total length

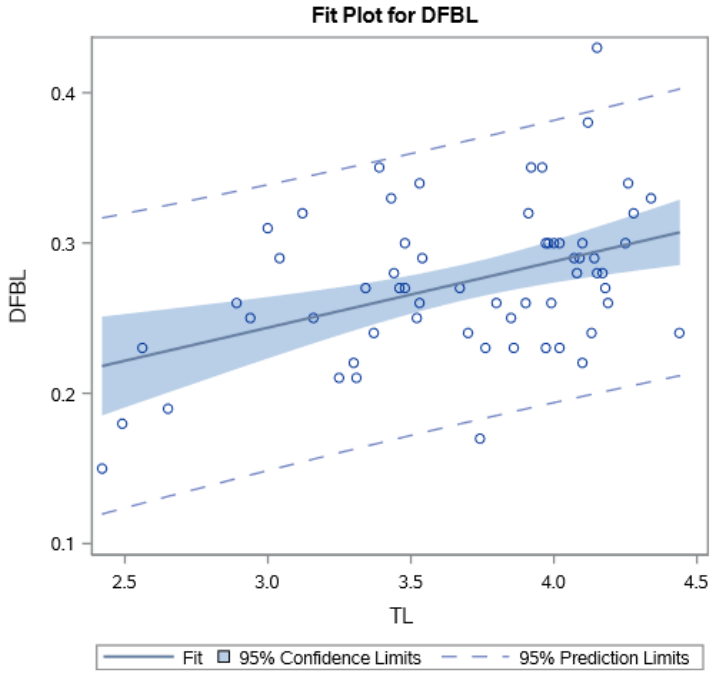

Fig.23.Regression curve of dorsal fin base length on total length

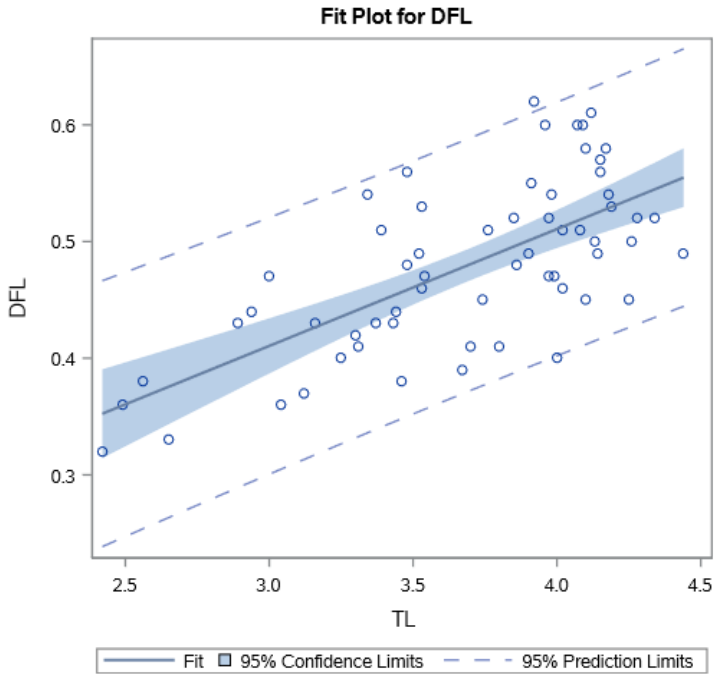

Fig.24.Regression curve of dorsal fin length on total length

4.5. Properties of selected microsatellite loci

The full length sequences with 1000 bp flanking regions at both ends for the six microsatellite loci (Shimoda *et al*., 1999) reported in ZFIN (https://zfin.org/) were retrieved in FASTA format from the NCBI GenBank. The sequences were individually subjected to Ensembl BLAT (BLAST Like Alignment Tool) analysis against the zebrafish (GRCz11) genome database at Ensembl genome database. The coordinates of each microsatellite locus and their location (non-coding, intronic, exonic or exon-intron boundaries) were determined. Information was also recorded about genes spanning the locus or those in the vicinity. The structure of repeat region was determined by Microsatellite Repeat Finder tool.

Table 7: Microsatellite loci Properties

Sl no.	Locus	LG	Genomic coodinates	Functional location	Details	Type & Repeats
1.	Z20576	7	7:55541901-55542306	Intron	aprt 203	Tri(ATC)12
2.	Z7141	1	1:27151452-27151740	Intron	bnc2	Di(TG)37
3.	Z7156	11	11:1354958-1355109	Intron	iars2	Di(AC)13
4.	Z10215	11	11:196831-197139	Non-coding	_	Di(TC)17
5.	Z20966	3	3:26824920-26825098	Exon	Clecl6a	Di(CA)13
6.	Z1547	3	3:6072556-6072739	Non-coding	_	Di(GT)11

arpt: adenine phospho ribosyl transferase

bnc2: Zinc finger protein basonuclin-2

iars2: isoleucyl-tRNA synthetase 1

Clec16a: C-type lecithin domain containing 16A

4.6. Microsatellite DNA analysis

4.6.1. PCR amplification of selected microsatellite loci

Specific primers designed earlier were used to amplify these loci. Genomic DNA extracted from zebrafish was subjected to PCR amplification using these 6 primer pairs. The PCR conditions were as mentioned earlier and the annealing conditions were provided in Table2.The amplified DNA product with the expected size (bp) is represented in fig 26 to 31.

4.6.2. Estimation of Genetic diversity in *D. rerios*

Fin tissue samples were collected from zebrafish as detailed earlier and genomic DNA was isolated from 23 individuals. The six primers (Primers 1-6 in Table 2.) were used to amplify specific loci from these genomic DNA samples. The amplified products were separated by PAGE on 10% gels and stained by ethidium bromide. The tracking dye was allowed to run out and gels were run for enough time to allow the bands to reach at least two thirds the length of the gel. The gels (Fig.32 to 37) were analysed by 'My image analyse software' (Thermoscientific) to determine the band sizes and individual genotypes were recorded. The number of alleles observed for each primer ranged from 2 to 7.

The mean observed number of alleles for overall population was 4.0. The highest effective number of alleles was 5.53 in Z7156 locus and the lowest effective number was 1.189 for Z10215 locus. The overall mean number of alleles, effective number of alleles, observed heterozygosity, expected heterozygosity and fixation index were 4.0, 3.259, 0.67, 0.585 and 0.154 respectively. The summary of Chi-Square Tests for Hardy-Weinberg Equilibrium reveals significant difference between the observed and expected heterozygosity and hence the markers deviate from Hardy-Weinberg Equilibrium.

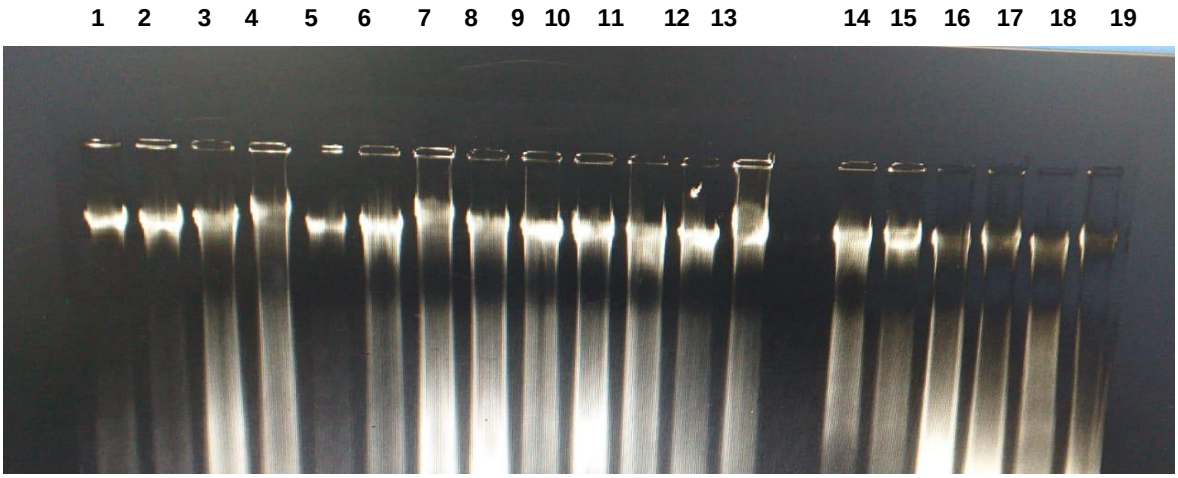

Fig 25: Lane 1-19 showing the isolated genomic DNA for 1% agarose gel

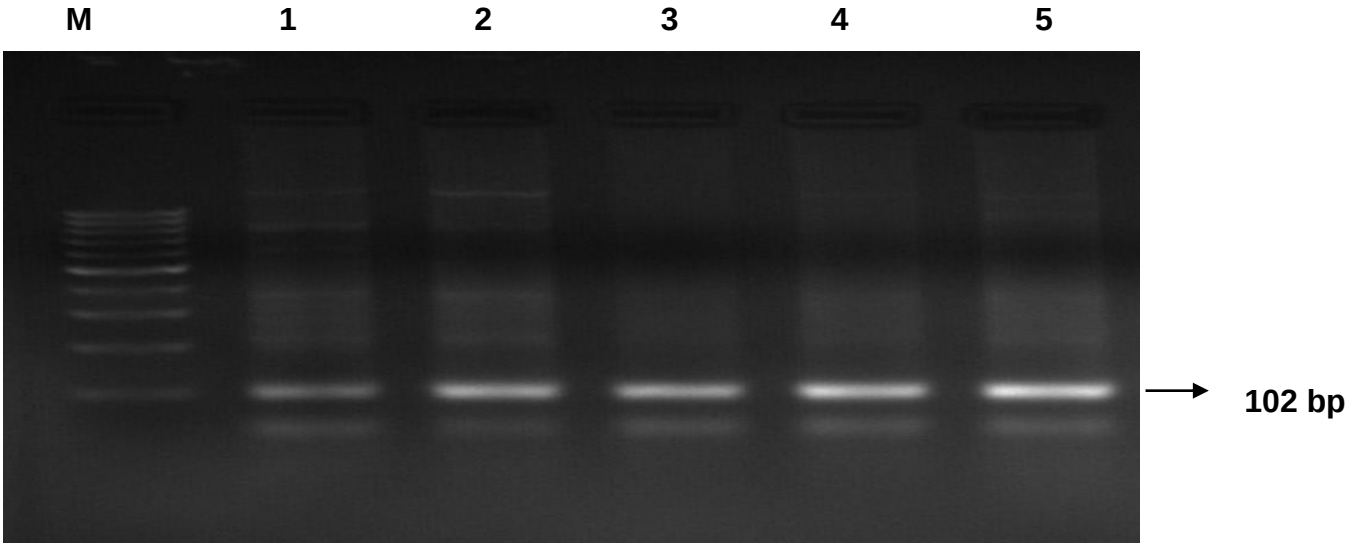

Fig 26: Locus name Z20576
Agarose Gel: Lane M: 100 bp DNA ladder, Lane 1 to 5: PCR product (102 bp)

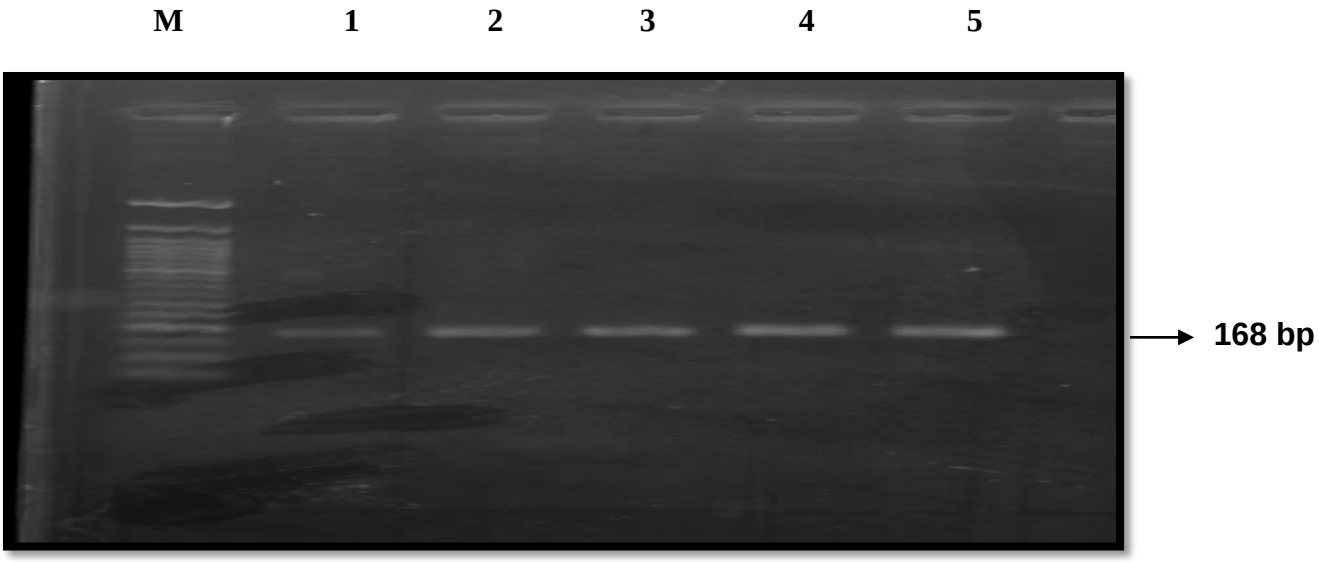

Fig 27: Locus name: Z7141
Agarose Gel: Lane M : 100 bp DNA ladder, Lane 1 to 6: PCR product (168 bp)

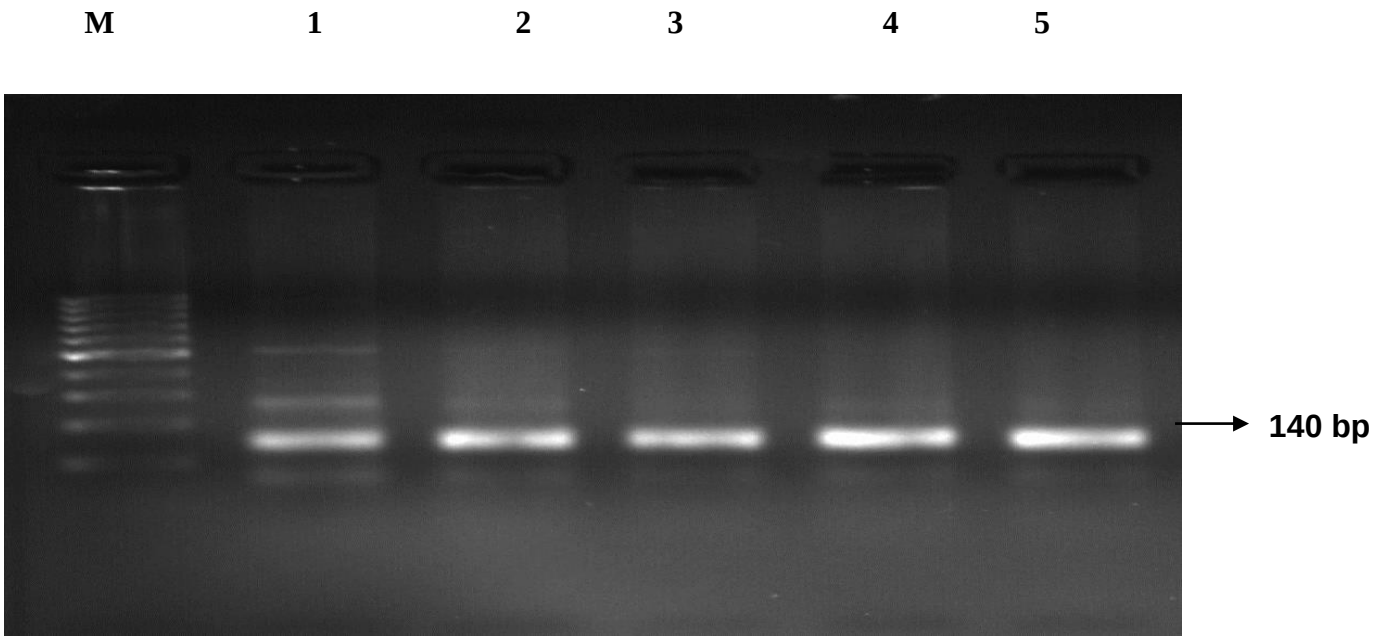

Fig 28: Locus name: Z7156
Agarose Gel: Lane M:100bp DNA ladder, Lane 1 to 5: PCR product (140bp)

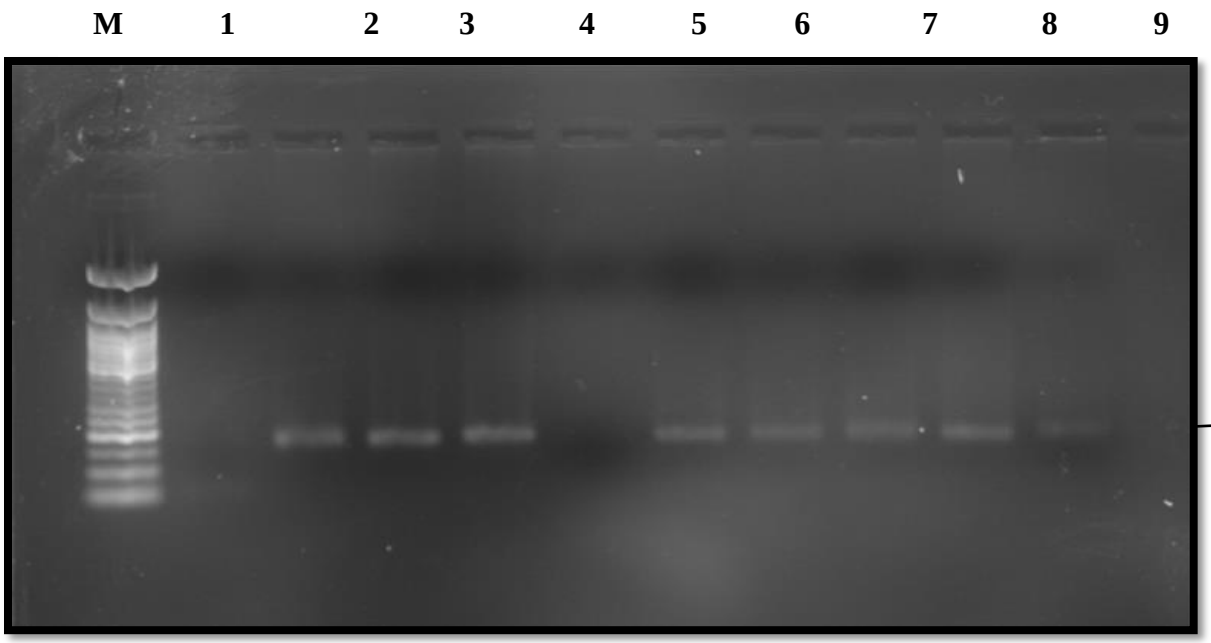

Fig 29: Locus name: Z10215
Agarose Gel: Lane M : 100 bp DNA ladder, Lane 1 to 11: PCR product (186 bp)

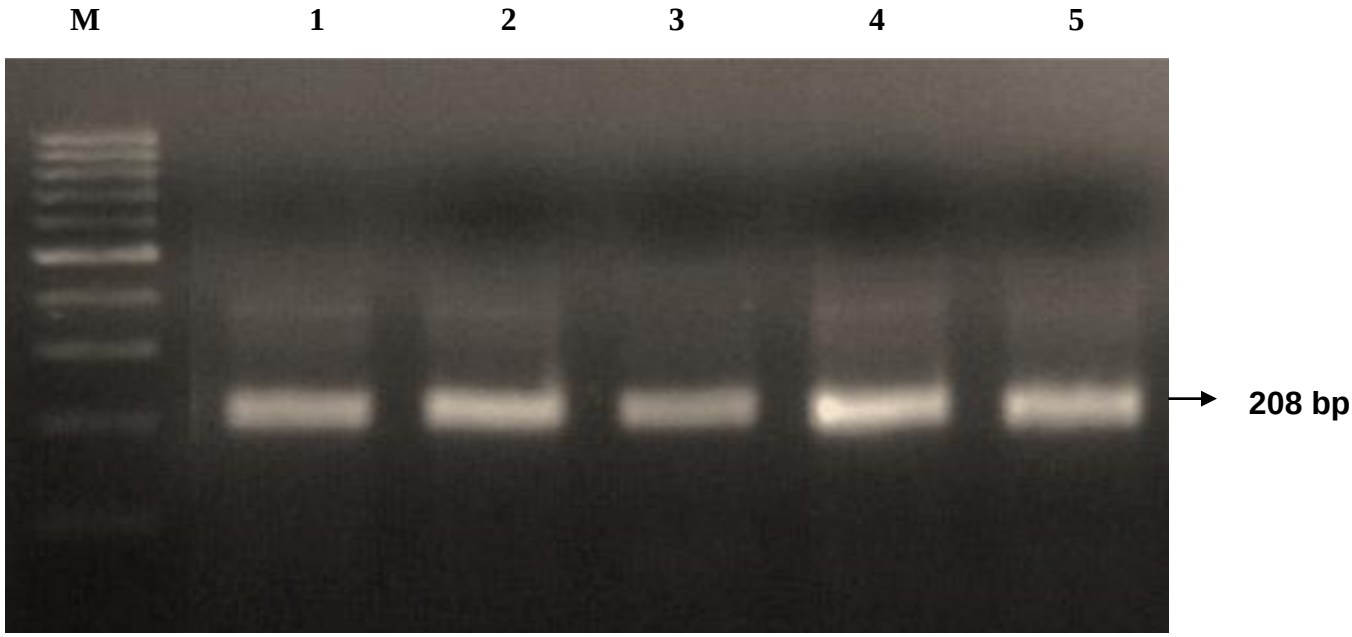

Fig 30: Locus name: Z15457
Agarose Gel: Lane M : 100 bp DNA ladder, Lane 1 to 5: PCR product (208 bp)

M 1 2 3 4 5

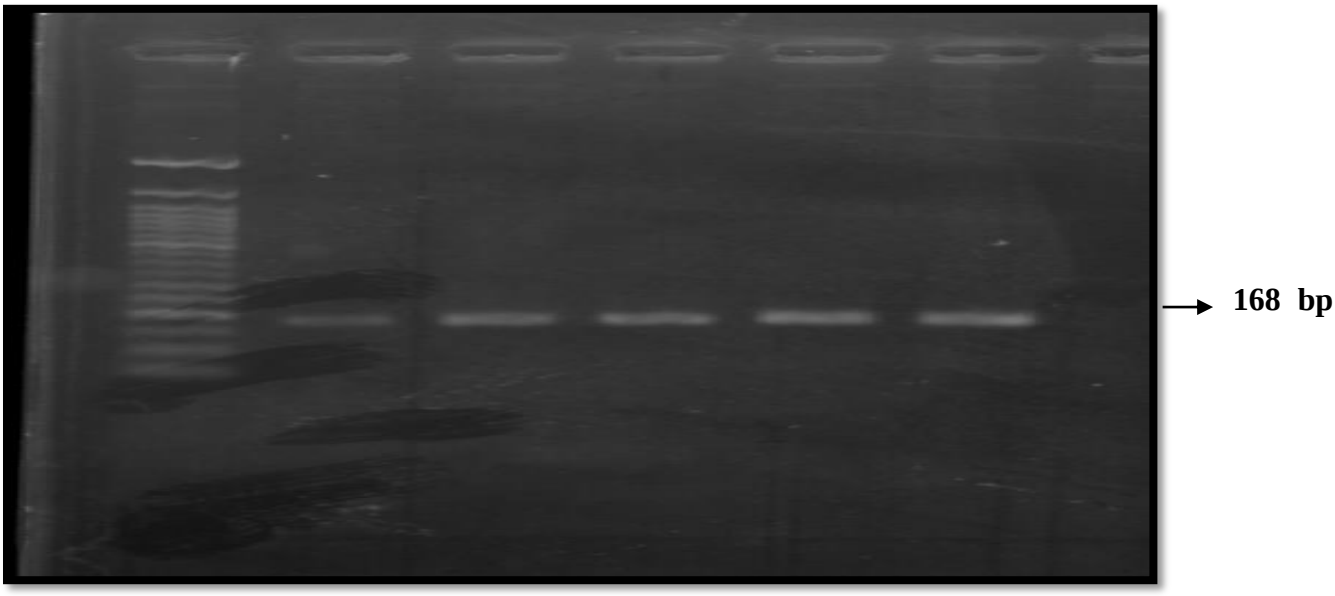

Fig 31: Locus name: Z20966
Agarose Gel: Lane M : 100 bp DNA ladder, Lane 1 to 5: PCR product (168bp)

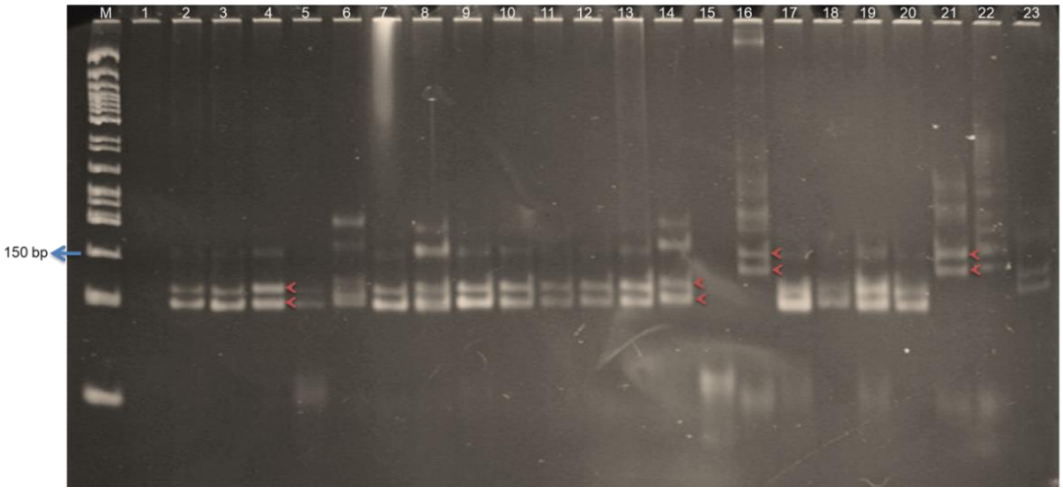

Fig 32: Allele distribution at locus Z20576 of *Danio rerio*. Lane M: 50 bp marker; Lane 1-23*: D.rerio* individuals. Allele size varies from 99 – 150 bp (4 alleles: 99,102, 144 and 150 bp) were observed at this locus

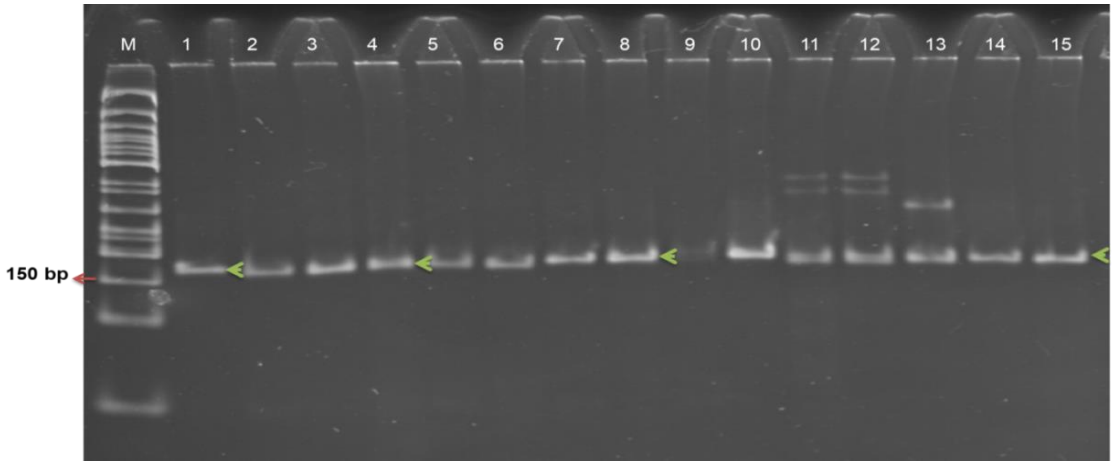

Fig 33: Allele distribution at locus Z7141 of *Danio rerio*, Lane M: 50 bp marker; Lane 1-15*: D.rerio* individuals. Two alleles (168 and 172 bp) were observed at this locus

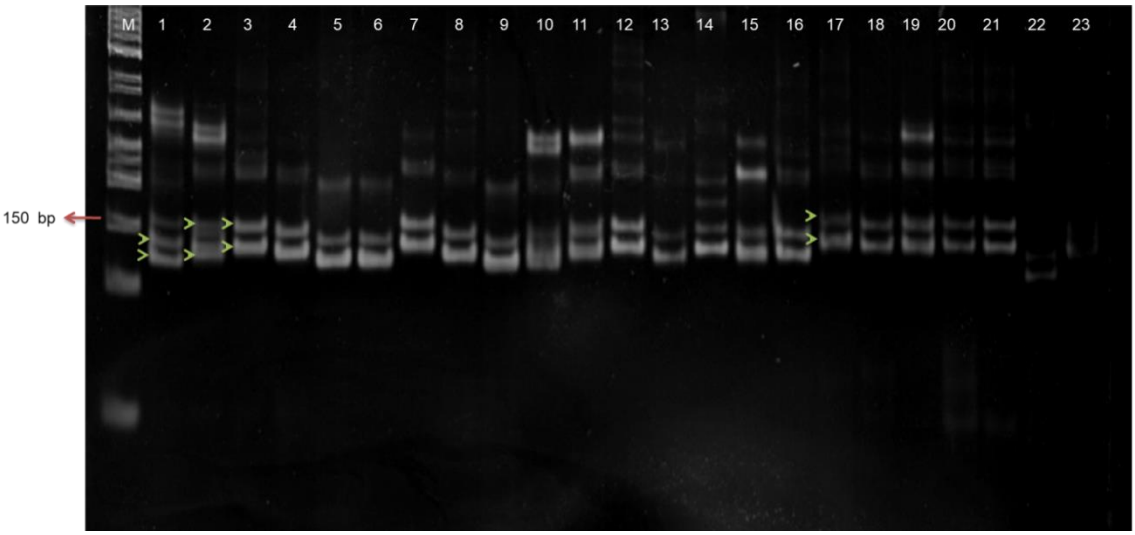

Fig 34: Allele distribution at locus Z7156 of *Danio rerio*, Lane M: 50 bp marker; Lane 1- 23*: D.rerio* individuals. . Allele size varies from 100 – 144 bp (7 alleles: 100,110, 114, 120, 130, 140 and 144 bp) were observed at this locus.

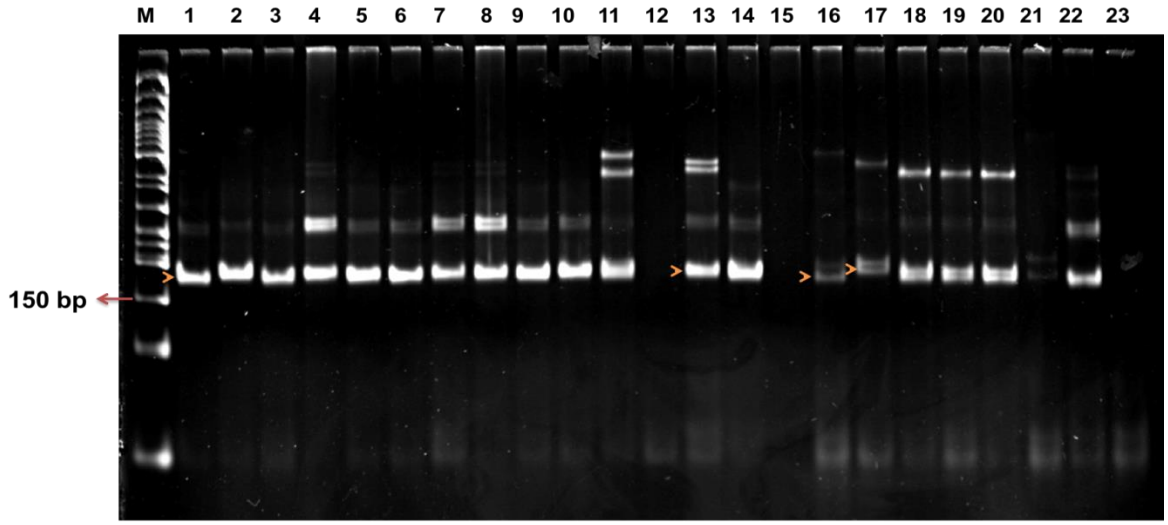

Fig 35: Allele distribution at locus Z10215 of *Danio rerio*, Lane M: 50 bp marker; Lane 1-23*: D.rerio* individuals. Two alleles (190 and 198 bp) were observed at this locus

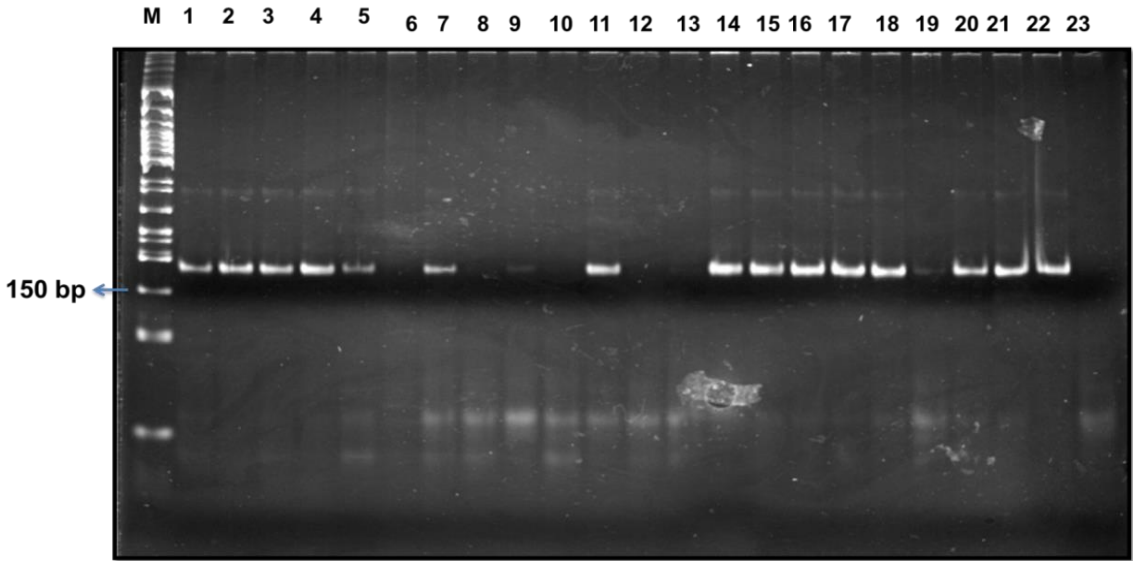

Fig 36: Allele distribution at locus Z20966 of *Danio rerio*, Lane M: 50 bp marker; Lane 1-23*: D.rerio* individuals. Allele size is 190 bp (monomorphic loci)

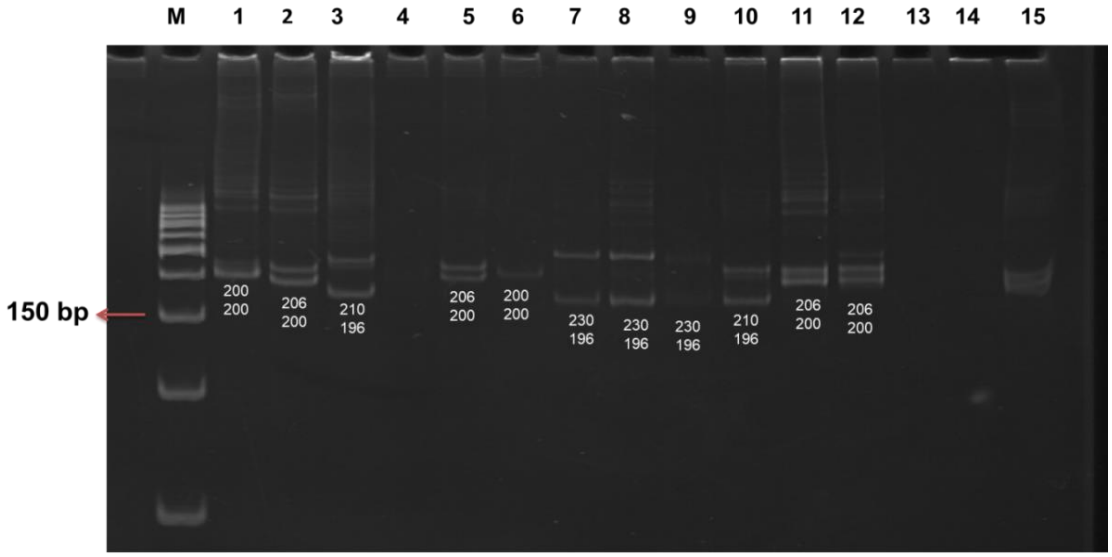

Fig 37: Allele distribution at locus Z15457 of *Danio rerio*. Lane M: 50 bp marker; Lane 1-23*: D.rerio* individuals. Allele size varies from 196 – 230 bp (5 alleles: 196,200, 206, 210 and 230 bp) were observed at this locus

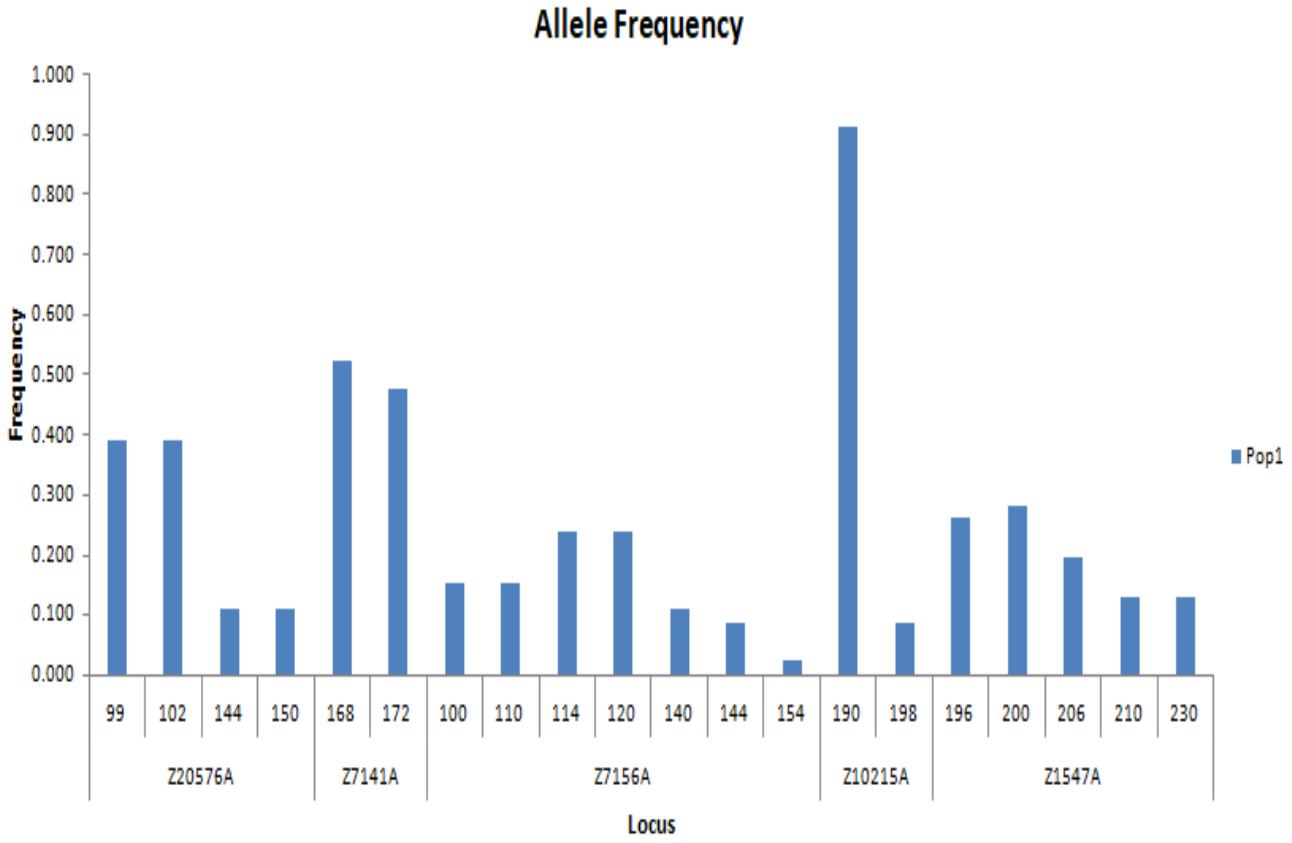

Fig.38. Allele Frequency Graph

Table 8: Characteristics of five Microsatellite markers

Locus	N	Na	Ne	I	Ho	He	uHe	F
Z20576	23	4.000	3.032	1.217	1.000	0.670	0.685	-0.492
Z7141	23	2.000	1.966	0.692	0.174	0.499	0.510	0.652
Z7156	23	7.000	5.539	1.794	1.000	0.819	0.838	-0.220
Z10215	23	2.000	1.189	0.295	0.000	0.159	0.162	1.000
Z1547	23	5.000	4.541	1.558	0.913	0.780	0.797	-0.171

Na = No. of Different Alleles

Ne = No. of Effective Alleles = 1/ (Sum pi^2)

I = Shannon´s Information Index = -1*Sum (pi*Ln (pi))

Ho = Observed Heterozygosity = No. of Hets / N

He = Expected Heterozygosity = 1- Sum pi^2

µHe = Unbiased Expected Heterozygosity = (2N / (2N / (2N-1)) * He

F = Fixation Index = (He- Ho) / He = 1 – (Ho / He)

Where, pi is the frequency of the ith allele for the population and sum pi^2 is the sum of the squared population allele frequencies.

Table 9: Overall characteristics of microsatellite markers

	N	Na	Ne	I	Ho	He	uHe	F
Mean	23.000	4.000	3.259	1.111	0.617	0.585	0.598	0.154
SE	0.000	0.949	0.799	0.276	0.219	0.120	0.123	0.285

Table 10: Summary of chi square tests for Hardy Weinberg Equilibrium

Locus	DF	Chisq	Prob	Signif
Z20576	6	69.000	0.000	***
Z7141	1	9.763	0.002	**
Z7156	21	115.000	0.000	***
Z10215	1	23.000	0.000	***
Z1547	10	57.024	0.0s00	***

ns= not significant, *P<0.005, **P<0.01, ***P<0.001

DISCUSSION

5. Discussion

The studies on morphological differences among populations from different ecological regions help towards understanding diversification of traits and are a fundamental step in adaptive radiation and ultimately, speciation (Shukla and Bhat, 2017). In India, the zebrafish is widely distributed in different river stretches and regions such as Brahmaputra, Gandak, Rapti, Karnal, Kali, Ramganga, Ganges, Yamuna, and Indus river drainages, Nadhave and Kalauma rivers in Kumaon Himalayas, Balangi district, Orissa Western Ghats, Kabini River, Thunga River and Wynaad district of Kerala (Menon., 1962, Hora., 1937, Chauhan., 1953 andTilak., 1968., Parichy, 2015).

The morphometric techniques have been applied to quantitatively separate individuals by shape (Mojekwu *et al*., 2015 and Zelditch, 2004). As a collection of statistical analyses on shape variables, morphometric examine the relationships of consistent landmark points between specimens (Adams *et al*., 2004). Techniques like geometric morphometric are gaining popularity (Zelditch, 2004) and have been applied to crustaceans (Accioly *et al*., 2013, Marchiori *et al*., 2014) insects (Orlofske and Baird, 2014), birds (Foster *et al*., 2008 and Shao *et al*., 2016) and fish (Silva, 2003 and Higham *et al*., 2017). Morphometric techniques has been effective in distinguishing sexes (Conradsen and McGuigan, 2015, Balazadeh and Litvak, 2018, Cadrin and Silva, 2005), geographical ranges (Silva, 2003) and strains (Varian and Nichols, 2010) for ecological and phylogenetic studies (Franssen *et al.,* 2015) of fish populations (Cadrin, 2000).

In the present study, twelve morphometric traits of zebrafish collected from Arunachal Pradesh were chosen viz; Total Length(TL),Standard length (SL), Caudal peduncle length (CPD), Maximum Body Depth (MBD), Eye diameter (ED), Pre-Opercular length (POL), Distance between upper jaw tip and ventral tip of operculum (UJVO), Anal fin base length (AFLB), Anal fin length (AFL),Caudal fin length (CFL),Dorsal fin base length (DFLB),Dorsal fin length (DFL). There was variation for all traits between and within the sex of zebrafish. Similarly morphometric studies were conducted by Shukla and Bhat, (2016). They quantified the extent of

differences in morphological traits between four populations of wild *D. rerio* from still-water (stagnant) (Kalibazaar, in West Bengal) and slow -medium flowing (Asan, Kaushalya and Seripetkalwa in Uttarakhand, Haryana and Andhra Pradesh respectively) habitats within India. The Caudal peduncle, Eye Diameter, Anal fin base length, and dorsal region between head and snout were observed in zebrafish. Zebrafish from stagnant water of Asan and Kaushalya had deeper bodies, deeper caudal peduncle and longer fins whereas zebrafish collected from slow-medium flowing water of Kalibazar had narrower caudal peduncle and shorter fins, due to the adaptations to different ecological conditions (Shukla and Bhat, 2016).Their study showed strong evidences for phenotypic variations in *D. rerio* populations.

The habitat types of *Danio rerio* in various streams/ rivers, secondary channels, oxbow lakes, and ponds, and irrigation canals connected to paddy fields from the Indian states of Kerala, Karnataka, Orissa, Uttar Pradesh, Meghalaya, Assam, and Arunachal Pradesh have been reported (Arunachalam *et al.,* 2013). Among the 21 populations, studied the individuals from two populations (one from Orissa and another from Arunachal Pradesh) were much larger in size (total length) when compared to other populations. The study reported that the zebrafish samples collected from Arunachal Pradesh belonged to Dikrong River at Khola camp, Rani village–Siang, Thore—Siang and Hapoli village–Sora River (Arunachalam *et al*., 2013). The total length of zebrafish ranged from 2.5 cm to 3.6 cm and it is similar with the zebrafish samples used in the current study. In our present study the zebrafish were collected from Ziro valley of Arunachal Pradesh. The overall total length observed was 3.69 ± 0.06 cm and the average value for female and male zebrafish was 3.88±0.07cm and 3.34±0.08 cm respectively. The morphometric measurements recorded for standard length, maximum body depth, and caudal peduncle length were also similar to the earlier reported studies.

Zebrafish exhibit a laterally compressed fusiform body shape, with fertile females being more ventrally rounded compared with the more slender males (Spence *et al*., 2008). Although both sexes carry the longitudinal patterning of

coloured stripes, males have a larger and more yellow tinged anal fin (Spence *et al*., 2008). It has also been reported that females possess an easily identifiable genital papilla (Yossa *et al*., 2013) and that pectoral fin rays are thicker in males due to more prominent breeding tubercles (McMillan *et al*., 2013 and McMillan *et al*., 2015). The experienced researchers can readily identify males and females that express these morphological traits (Duff *et al*., 2019). However, these traits are not always easily seen or expressed, making identification of these individuals difficult and vulnerable to observer experience (Duff *et al*., 2019). Everyday tasks such as managing broodstock facilities, maintaining appropriate sex ratios, and selecting individuals for breeding pairs require development of more accurate and precise markers of sex in zebrafish (Duff *et al*., 2019).

Zebrafish (*Danio rerio*) adults are viewed as sexually dimorphic. However, current approaches to sex discrimination rely mainly on subjective assessment of colour patterns and body structures. In the present study, it was observed that the female sex of zebrafish measured significantly higher for total length, standard length, caudal peduncle length, maximum body depth, pre-opercular length, anal fin base length and dorsal fin length but for traits *viz*; eye diameter, distance between upper jaw tip and ventral tip of operculum, anal fin length, caudal fin length and dorsal fin base length, there was no significant difference between female and male sex of zebrafish. The present study delineates quantitative differences between sexes of zebrafish but further studies need to be conducted for quantitative sex discrimination. Duff *et al.*, (2019) explored how geometric morphometric allow for quantitative sex discrimination based on overall body geometry of adult zebrafish (aged 12–24 months). Analysis of body geometry demonstrated that males have a longer caudal peduncle, a more streamlined ventral region, and slightly more inferior placement of eyes than females. They developed a logistic regression equation using the ratio of ventral caudal peduncle length to standard length to provide researchers a reliable and objective method for sex discrimination in zebrafish.

In the present study, the morphometric traits of zebrafish were also analysed as function of total length using a simple linear regression equation. All the traits were positively correlated with total length. This indicates that the growth of fish in one area of the body is co-related to growth in another area of the body. Different values of correlation coefficient indicate the varying relationships of total length with the parameters. Similar results were reported by Bhat *et al*., (2016), in *Cyprinus* sp. Khillare and Sonawane (2016) studied morphometric characteristics of *Mystus armatus* from Aurangabad region and all body parameters exhibited positive correlation. Brraich and Akhter (2015) also affirmed the positive correlation between body parameters and total length of minor carp, *Crossocheilus latius* collected from a wetland in India.

In the present study the overall mean number of alleles, effective number of alleles, observed heterozygosity, expected heterozygosity and fixation index were 4.0, 3.259, 0.67, 0.585 and 0.154 respectively. Coe et al. (2008) analysed wild zebrafish collected from Bangladesh andfound high variability, with an allelic richness of 14.126 and expected heterozygosity of 0.855. Wild zebrafish have previously been shown to be highly polymorphic and heterozygous at various microsatellite loci, with up to 35 alleles per locus (Gratton *et al.,* 2004). In four populations of wild zebrafish, allelic richness was between 9.307 and 11.463 and mean expected heterozygosity was between 0.737 and 0.802 (Gratton *et al.,* 2004). In the present study the microsatellite locus viz., Z20576, Z7141, Z7156 were located in intron regions of a gene and exhibited polymorphism whereas the locus Z20966 was located in exon region of a gene and was found to be monomorphic. Microsatellites have been used for decades as putatively neutral markers to study the genetic structure of diverse populations. However, recent studies have demonstrated that some microsatellites contribute to gene expression, *cis* heritability, and phenotype. The microsatellites have the capacity to affect gene expression and may be leveraged by natural selection for efficient evolution (Kinney *et al.,* 2019).Strong enrichments were found near transcription start sites and predicted enhancers (Willems *et al*., 2016).

SUMMARY

SUMMARY

The zebrafish, *Danio rerio*, Teleostei infraclass, Cyprinidae family, is a monophyletic group and has emerged as an excellent vertebrate model organism for studies related to genetics, immunology, developmental and cancer biology, etc. The zebrafish is native to freshwater habitats in South Asia (Lawrence, 2007). In India, it typically inhabits the moderately flowing top stagnant clear water of quite shallow depth in streams and canals of the Sutlej, Ganges, Brahmaputra, Chindwind river basins, Western Ghats and the Eastern Ghats. Arunachal Pradesh is the easternmost state of India and is a micro-hotspot within the larger eastern Himalayas. Ziro valley in Arunachal Pradesh is a picturesque valley that lies at an altitude of 1573 meter above mean sea level. Paddy-cum-fish cultivation is popular in this valley and the water available through irrigation canals connecting the streams. These irrigation canals connecting paddy fields form the main hub for Zebrafish (Kacha, 2016). Even though laboratory strains of zebrafish are subjected to intense research, scarce information is available with respect to wild zebrafish population size and structure. Study of variation in morphometric traits of zebrafish and use of microsatellite markers helps to assess genetic diversity in zebrafish.

About 61 zebrafish samples were collected from three different sites of Ziro valley.12 morphometric parameters sex-wise were recorded in zebrafish using ImageJ (version 1.51). The molecular analysis included, DNA isolation of all individuals, quality check and quantification of DNA by agarose gel electrophoresis on 1% agarose gel, PCR amplification using six microsatellite loci *viz*; Z20576, Z7141, Z7156, Z10215, Z20966, Z15457. The Z20576 was trinucleotide and the rest were dinucleotides. The locus Z20966 was monomorphic. Amplified products were separated by PAGE (Polyacrylamide gel electrophoresis), and the size of the amplicons of microsatellite loci (alleles) was estimated using DNA ladder by My Image Analysis software v2.0. GenAlex 6.4 software was used for statistical analysis of genetic parameters *viz*; allele frequency observed and expected heterozygosity values. Polymorphic information content (PIC) values were estimated for all loci by

Cervus v.3.0. Descriptive statistics and analysis of variance were analyzed by PROC MEANS procedure and PROC GLM of SAS® 9.4 system. A fixed model, $y_{ij}= \mu + gender_i + e_{ij}$ was adopted to study the least square means. Morphometric studies on adult zebrafish samples showed that the effect of sex was significant for most of the traits. The effect of sex was significant on total length, standard length and maximum body depth. However, traits such as eye diameter, the distance between upper jaw tip and ventral tip of the operculum, anal fin length, caudal fin length and dorsal fin length showed no significant sex-based difference. All the traits showed a positive correlation with the total length. The genetic diversity in zebrafish collected from Ziro valley was assessed and the number of alleles observed for each primer ranged from 2 to 7.The mean observed number of alleles for the overall population was 4.0. The highest effective number of alleles was 5.53 in Z7156 locus and the lowest effective number was 1.189 in Z10215 locus. The overall mean number of alleles, effective number of alleles, observed heterozygosity, expected heterozygosity, fixation index and PIC for the markers were 4.0, 3.259, 0.67, 0.585, 0.154 and 0.64 respectively. The morphometric studies revealed strong differences for traits between sexes. Further, geometric morphometric studies may help in quantitative sex discrimination in zebrafish. Four markers exhibited higher heterozygosity values and may be suitable for genetic characterization of zebrafish stocks in future.

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ANNEXURES

APPENDIX I

1. Equipment and Laboratory – Ware

- Glassware, Borosil, India
- Plasticware, TarsonsPvt. Ltd, India
- Disposable Syringe Filters; Osmonics, USA
- Thermo cyclers, BioRad (USA) and Applied Biosystems (USA)
- Nanodrop Spectrophotometer (USA)
- Refrigerated Centrifuge (Hereaus, Germany)
- Horizontal Gel Electrophoresis apparatus (Thermo, Scientific, USA)
- Gel documentation system, BioRad (USA)
- Electronic balance , OHAUS (USA)
- Table-top centrifuge 5430 (Eppendorf, Germany)
- Microwave oven (Kenstar, India)
- Autoclave and Micropipettes (Eppendorf, Germany)
- Autoclave demineralised water MilliQ

APPENDIX II

Reagent composition

1. Anaesthetizing of fish samples

1.5g/L stock solution of Ms-22, Sodium bicarbonate used to buffer the stock until p H of 7.2 – 7.4 recorded.

Absolute alcohol for sample preservation

2. Genomic DNA isolation

2.1. Phenol-chloroform method:

A) **TEN Buffer:**

0.1 M Tris-Cl (pH 8.0)	7.88gTris base
0.01 M EDTA (pH 8.0)	1.46gm EDTA
1 M NaCl	23.37gm NaCl

B) **10 % SDS**

10gm of Sodium Dodecyl sulphate in 100ml of distilled water

C) **Proteinase K** (20mg/ml):

Store at -20^{0}C.

D) **Chloroform: Isoamyl alcohol (24:1):**

Chloroform	24 ml
Isoamyl alcohol	1 ml

E) **TE Buffer:**

Tris-HCl (pH 8.0)	10 mM
Na2 EDTA. 2H2O (pH 8.0)	1 mM

F) **RNase (10mg/ml):**

RNase	10 mg
Sodium acetate (0.01M; pH 5.2)	1 ml

Heated to 100^0C for 15 minutes

3. Gel electrophoresis

3.1. EDTA (0.5 M; pH 8.0)

EDTA	18.61gm
DMW	80ml

pH adjusted to 8.0 with NaOH. Make up the volume to 100ml and sterilize by autoclaving.

3.2. Tris-Cl (1M; pH 8.0)

Tris base	12.11gm
DMW	100ml

pH adjusted to 8.0 with conc. HCl and sterilized by autoclaving.

3.3. TAE (50X)

Tris base	242gm
Glacial acetic acid	57.1ml
EDTA (0.5M; pH 8.0)	100ml

Final volume made to 1000ml.

3.4. Ethidium bromide (10 mg/ml)

Ethidium bromide 0.1gm

DMW 10ml

Covered with aluminium foil and stored at 4^0C.

3.5. Gel loading dye (6X)

Bromophenol blue 0.25gm

Xylene cyanol 0.25gm

Glycerol 3ml

Make the volume to 10ml using distilled water and stored at 4^0C.

4. Polyacrylamide gel electrophoresis

4.1. Acrylamide- Bisacrylamide Solution

Acrylamide (30%)	33.3ml
5X TAE Buffer	20ml
10% APS (Prepared fresh)	700µl
TEMED	35µl
DMW	46ml (to make up to 100ml)

4.2. TBE buffer 10X (pH-8.0)

Tris base 108g

Boric acid 55g

In 800ml of distilled water

EDTA (0.5M) 40ml

Make the volume upto 1000ml with distilled water

Autoclave and store at room temperature

4.3. Gel loading buffer

Bromophenol blue 0.5%

Glycerol (molecular grade) 30%

Prepare in 1X TBE

Store at 4^0C

4.4. 1X TBE buffer

10X TBE 10ml

Distilled water 90ml