RNA-Seq Transcriptome Profiling of Ethiopian Lowland Bamboo (*Oxytenanthera Abyssinica* (A.Rich) Munro Under Drought and Salt Stresses and SSR Based Genetic Diversity Analysis o f Ethiopian Highland Bamboo (*Arundinaria Alpina* K. Schum).

Muhamed Adem

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LIST OF ABBREVATIONS and ACRONYMS

AMOVA	Analysis of molecular variance
BLAST	Basic local alignment search tool
BPG	Bamboo phylogeny group
cDNA	Complementary DNA
COG	Clusters of Orthologous groups
DEGs	Differentially expressed genes
FAO	Food and Agricultural Organization
FDR	False discovery rate
FPKM	Fragment per kilo bases per million reads
GBS	Genotyping by sequencing
GO	Gene ontology
He	Expected heterozygosity/Nei's gene diversity
Ho	Observed heterozygosity
HWE	Hardy-Weinberg equilibrium
I	Shannon information index
ICBR	International center for bamboo and rattan
INBAR	International network for bamboo and rattan
ISSR	Inter simple sequence repeats
KEGG	Kyoto encyclopedia of genes and genomes
MiRNA	Micro RNA
mRNA	Messenger RNA
Na	Number of alleles
NCBI -Nr	National center for biotechnology information non-redundant
Ne	Number of effective allele
NGS	Next generation sequencing
NJ	Neighbor joining
NTFPs	Non-timber forest products
*Oa*bZIP	*Oxytenanthera abyssinica* bZIPs
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PEG	PolyEthylene glycol
PlantTFDB	Plant transcription factor database
qRT-PCR	Quantitative real time-PCR
QTL	Quantitative trait loci
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species

SNP	Single nucleotide polymorphism
SNPPR	Southern Nations, Nationalists and Peoples' Region
SSR	Simple sequence repeats
TFs	Transcription factors
tRNA	Transfer RNA
WUE	Water use efficiency

ABSTRACT

RNA-Seq transcriptome profiling of Ethiopian lowland bamboo (*Oxytenanthera abyssinica* (A.Rich) Munro under drought and salt stresses and SSR based genetic diversity analysis of Ethiopian highland bamboo (*Arundinaria alpina* K. Schum)

Muhamed Adem Munie

Addis Ababa University

Bamboos are perennial grasses classified under family Poaceae and subfamily Bambusoideae and are among the fastest growing plants on earth. Although bamboos have ecological and economic importance in Ethiopia, scientific inquiry particularly on genomics, transcriptomics and genetic diversity and structure is lacking. Ethiopian lowland bamboo (*O. abyssinica*) adapted in hot areas of Ethiopia hence inquiry on global transcriptome profiling is very important to elucidate the most important genes, transcript factors and metabolic pathways associated to abiotic stress. To do so, plastic pot germinated seedlings of lowland bamboo were subjected to 200 mM NaCl and 25% PEG-6000 (Poly Ethylene glycol) to induce salt and drought stress, respectively. Using the Illumina sequencing platform, fifteen cDNA libraries were constructed and sequenced to generate the first drought and salt stress global transcriptome profiling of the species. Following quality control, 754,444,646 clean paired-ends reads were generated, and then *de novo* assembled into 406,181 unigenes. Functional annotation against the public databases presented annotation of 217,067 (53.4%) unigenes, where NCBI-Nr 203,777, Swissport 115,741, COG 81,632 and KEGG 80,587. Prediction of Transcripts Factors (TFs) has generated 4,332 TFs organized into 64 TF families. Analysis of Differentially Expressed Genes (DEGs) provided 65,471 genes where 569 genes belongs to all stresses. Transcript factors (TFs) with a higher number of differentially expressed genes include bZIP (49), WRKY (43), MYB (38), AP2/ERF (30), HD-ZIP (25) and MYB related (21). Such 569 genes could serve for engineering plants for multiple abiotic tolerances. Despite the important role of bZIPs in plants, particularly in abiotic tolerances, bZIP family members and their corresponding functions remain elusive in the lowland bamboo. Through genome-wide analysis, a total of 162 bZIP lowland bamboo TFs having the bZIP binding domain (PF00170) were identified. For the identified bZIPs, functional annotation, phylogenetic relationship and their expression under drought

and salt stress were investigated. The expression profile of the bZIP TFs revealed that majority of the bZIPs were highly responsive to drought and salt stress as 99 of the 162 TFs were up-regulated. Metabolic pathway analysis revealed that environmental information processing and genetic information processing categories were the only represented pathway which implies that the bZIPs are associated to drought and salt stress.

Understanding on the genetic diversity and structure of highland bamboo is important particularly for conservation. For genetic diversity analysis 150 samples, representing 15 populations, 10 samples from each population were collected. The sources of SSR primers were; first, primers developed through reduced-representation genome sequencing of lowland bamboo were checked for their polymorphism and transferability, then 7 primers were selected and used for highland bamboo. Second, primers were directly taken from literature from Chinese Moso bamboo, after checking their transferability 9 primers were used. The genetic diversity indices (H= 0.398 and I= 0.639) for the 16 SSR markers used were moderate in capturing genetic variability. Both individual phylogeny and population structure merged the 15 populations in to nine clusters, with less geographic origin or collection based pattern. UPGMA clustered the 15 populations in 2, while NJ clustered in to 3, however pattern of their grouping is not in line with their pattern of origin of collection. The principal component analysis revealed that individuals are highly dispersed and did not group the populations into stridently distinct clusters which tells the presences of high genetic admixture possibly attributed to gene flow or the reproductive biology of the species. In conclusion, the RNA-Seq based global transcriptome profiling has generated transcriptomics resources for lowland bamboo which can be employed for itself and related species for further research. Most importantly the study uncovered key stress responsive genes, transcription factors, metabolic pathways and even genes differentially expressed to all stress groups which could be used as the basis for further studies aiming to confer plants for multiple abiotic stress tolerances. The study on bZIPs genome wide analysis also provided valuable information on how bZIPs are highly associated to abiotic stress tolerance since only pathways related to stress tolerances were represented in metabolic pathway analysis. The genetic diversity and population structure analysis of highland bamboo uncovered the presences of moderate genetic variability with high genetic admixture. The information could be used as a basis particularly for conservation interventions and for further investigations.

Key Words: *Arundinaria alpina*, Abiotic stress, differentially expressed genes, genetic diversity, *Oxytenanthera abyssinica*, RNA-Seq, SSR, Transcriptomics

CHAPTER 1

1. GENERAL INTRODUCTION

Bamboos are among the most economically and environmentally valuable plants on earth. There are 75 genera and approximately 1,250 species of bamboos distributed in a range of environments from tropical and warm ecosystems to cold regions. The majority of the species and species richness are restricted to Asia, (mainly China: 626 species, India: 102, Japan: 84, Burma: 75 and Malaysia: 50), Central and South America, (mainly Brazil: 134, Venezuela: 68 and Colombia: 56) and 43 species in Africa (Chaomao *et al*., 2006; Das *et al*., 2008). Majority of the African 43 bamboo species grows only in Madagascar (Bekele, 2007). Other African countries with bamboo resources include Tanzania, Malawi, Ethiopia, Uganda, and Zambia. Only two species of Bamboos, namely *Arundinaria alpina* K.Schum and *Oxytenanthera abyssinica* (A.Rich) Munro are found in Ethiopia. Due to its superior wood substitute, cheap, efficient, fast-growing and a high potential for environmental protection and wide ecological adaptation, bamboo is one of the world's most important Non-Timber Forest Products. Now a day, worlds' forest resource are shrinking and thus potential alternative species is wanted (Ohrnberger, 1999; Mekonen *et al*., 2014).

Bamboo undergoes a 'fast stem growth' phase elongating as high as about 91 cm in a span of 24 h (Fu, 2001). The world record for the fastest growing plant belongs to certain species of the 45 genera of bamboo, which have been found to grow at up to 91 cm (35 in) per day or at a rate of 0.00003 km/h (0.00002 mph) accessed online at (https://www.guinnessworldrecords.com/world-records/fastest-growing-plant/) .

Plant growth and development is hampered by many environmental factors. Sessile nature of plants makes them unable to distance themselves from unfavorable conditions. To survive in harsh conditions plants should develop adaptive mechanisms like a synthesis of functional proteins with different functions (Hu *et al*., 2013; Llorca *et al*., 2014). In recent decades, thousands of genes and dozens of metabolic and signaling pathways actively involved in plants response to abiotic stresses have been identified and characterized (Zhou *et al*., 2007; Molina *et al*., 2008). The roles of some genes in improving plant tolerance to drought and salt stress have been well confirmed (Hussain *et al*., 2011).

Presently, next-generation sequencing (NGS) based RNA sequencing has been widely used to decipher various abiotic responses in many plants especially for those functionally not annotated species (Wang *et al*., 2009). Mainly due to their high accuracy, sensitivity, efficiency, low cost and throughput, next-generation sequencing (NGS) technologies revolutionized the transcriptomics studies (Buerman and Dunnen, 2014). In the light of this, NGS has been widely used to sequence *de novo* assembled transcriptomes in many plants like Moso bamboo, Ma bamboo, Poplar, European beech, Buckwheat (Zhao *et al*., 2016; Yao *et al*., 2017; Muller *et al*., 2017; Wu *et al*., 2017).

Transcription factors (TFs) consist of sequence-specific DNA binding domain for binding to the promoter and/or enhancer regions of corresponding genes, thereby inducing or repressing transcription of downstream target genes (Balogu *et al*., 2014). Transcription factors (TFs) are responsible for regulating gene expression through binding to cis-regulatory specific sequences in the promoters of their target genes.

As bamboos are polyploid plants their genetical studies are complicated. Due to this reason, taxonomic and diversity studies were conducted on the basis of vegetative and floral characteristics. But, because of a long time gap in flowering, characterization of bamboo via conventionally used flower characters resulted in confusion of bamboo taxonomy grouping. Vegetative characters which are subjected to change to environmental factors are also not reliable for bamboo systematic grouping. In addition, protein markers are not reliable in revealing the existing genetic diversity when compared with molecular markers (Embaye, 2000). Molecular markers are not subjected to change with environmental factors have been developed for the precise study of plant taxonomy.

Furthermore, molecular approaches are robust in identifying genes of economic and biotechnological interest and characterizing the genetic diversity among different species. Molecular markers, particularly Simple Sequence Repeats (SSRs), are among the markers that are widely used in plant genetic diversity study, due to their co-dominant nature, high reproducibility and abundance in the genome (Zhao *et al*., 2015; Attigala *et al*., 2017; Cai *et al*., 2019). But the first problem in using SSRs in molecular research is the cost and effort required for sequencing and designing of species-specific primers for amplification of locus-specific SSRs (Squirrell *et al*., 2003). This hampers the use of SSR for plant species with no or limited genomic information. Sequence data obtained from several plant species indicate sufficient homology between genomes in the regions harboring microsatellites (Satya *et al*.,2016). An alternative approach is to search for SSR markers transferable to closely related species or genera. But, for species with no searchable SSR, the remaining option is to sequence and develop SSR markers (Kozich *et al*., 2013).

Besides, the advent of low-cost next-generation sequencing technology, reduced-representation genome sequencing (RRGS) has simplified genotyping (Sun *et al*., 2013). RRGS, became an alternative to complete genome sequencing as it is capable of generating fast and accurate results and inexpensive sequences data for complex and large genomes like Bamboo (Barbazuk *et al*., 2005). For SSR marker discovery using RRGS, only those genomic regions with repetitive sequences are selectively sequenced (Kozich *et al*., 2013).

Microsatellite markers, also known as simple sequence repeats (SSR), are important in studying plant population genetic structure, genome mapping, cultivar identification, genetic diversity and evolutionary genetics. SSR are preferred since they have wide genome coverage, relative abundance, amenability to automation, high information content, polymorphic and co-dominant (Powell *et al*., 1996; Kalia *et al*., 2011). In recent years, Microsatellite (SSR) markers have been developed for several bamboo species. These markers have been applied to estimate genetic diversity; population differentiation and population structure for the species itself and for related ones (Miyazaki *et al*., 2009; Jiang *et al*., 2013; Lin *et al*., 2014; Jiang *et al*., 2017).

1.1 Statement of the problem

Despite the good potential in offering economic development and ecological benefits, researches on various aspects of bamboos are receiving less attention from the scientific community. Some of the research activities conducted on Ethiopian bamboos so far are limited to ecological and resource management of highland bamboo (Embaye, 2000; Embaye, 2001; Embaye *et al*., 2003; Embaye *et al*., 2005), suitability of highland bamboo for oriented particle board (Kelemework , 2005; Kelemework *et al*., 2007), utilization of lowland bamboo stems as reinforcement steel

(Melaku Abegaz *et al*., 2005); vegetative propagation of highland bamboo (Hunde *et al*., 2005), adaptation and growth performance of lowland bamboo (Terefe *et al*., 2006) and biology, ecology and silvicultural management of highland bamboo (Mulatu, 2012).

Although the above research activities have been done so far, there are still many research gaps that have to be addressed. Among the information still lacking are; 1, Drought and salt stress-induced RNA-Seq global transcriptome profiling of lowland bamboo, 2, genome-wide analysis and expression profiling of bZIPs, the most important transcript factor gene family associated with drought and salt stress and 3, extent of genetic diversity and population structure within and among highland bamboo populations of Ethiopia. Therefore, the objective of this study was to overview the global transcriptome alternation of lowland bamboo under drought and salt stresses and to assess the genetic diversity of Ethiopian highland bamboo.

1.2 Hypothesis

The following null hypotheses have been stated;

- ✓ There were no differentially expressed genes in response to drought and salt stress treatment of lowland bamboo.
- ✓ Lowland bamboo's bZIP transcript factor gene families expression pattern do not associate with drought and salt stress.
- ✓ Polymorphic SSR markers do not exist from reduced-representation genome sequencing of Ethiopian lowland bamboo and from SSR markers of Chinese Moso bamboo (Phyllostachys edulis) to Ethiopian highland bamboo
- ✓ There is no genetic diversity within and among highland bamboo populations of Ethiopia.

1.3 Objectives

1.3.1 General objective

To explore the transcriptome profiling of lowland bamboo in response to drought and salt stress and genome-wide analysis and expression profiling of bZIPs and to assess the extent of genetic diversity and population structure of highland bamboo populations of Ethiopia.

1.3.2 Specific objectives

1. To investigate drought and salt stress-induced global transcriptome profiling of lowland bamboo.

2. To understand the association of bZIPs gene families expression oscillation to drought and salt stress conditions.

3. To develop polymorphic SSR markers from reduced- representation genome sequencing of lowland bamboo and from already developed SSR markers from Chinese Moso bamboo.

4. To determine the level of genetic variation existing within and among highland bamboo populations in Ethiopia.

1.4 Organization of the thesis

The dissertation is outlined as follows:

Chapter One presents the general introduction of the dissertation which includes the background, statement of the problem and objectives of the study.

Chapter Two presents the literature review on bamboos, Ethiopian bamboos and their botanical descriptions, Ethiopian lowland bamboo (*Oxytenanthera abyssinica*), Ethiopian high land bamboo (*Arundinaria alpina*), RNA-Seq and transcriptomics, Plant abiotic stress, Plant

transcription factors, Basic leucine zipper (bZIP) transcription factor gene family, GO, COG and KEGG, Molecular markers and Microsatellite (SSR) markers.

Chapter Three deals with RNA-Seq global transcriptome profiling of Ethiopian lowland bamboo (*O.abyssinica*) under drought and salt stress conditions. This section of the work is published in the Open Biotechnology Journal. 2019 13: 6-17.DOI: 10.2174/1874070701913010006. This part of the work investigated the global transcriptome profiling of lowland bamboo under drought and salt stress. The most important genes, transcript factors gene families and metabolic pathways associated with drought and salt stress were identified.

Chapter Four presents the Genome-wide analysis and expression profile of bZIPs transcript factor gene family in lowland bamboo under drought and salt stress. This part is submitted to Journal of Applied Genetics and its under review status. Through transcriptome analysis, bZIPs were found to be with the highest number of protein-coding and differentially expressed genes. Due to this, detailed analysis of their identification, phylogeny, functional annotation and expression profiling of the bZIPs was conducted.

Chapter Five deals with the extent of genetic diversity and population structure of Ethiopian high land bamboo (*Arundinaria alpina*) populations as revealed by SSR markers. Understanding the extent of genetic diversity particularly using co-dominant markers like SSR is very important in designing proper conservation and utilization strategy for a given species.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Bamboos

2.1.1 Origin of bamboos

Definition of forest varies. But, according to FAO (2006) forest is defined as an area having a minimum land area of 0.5-1 ha with tree crown cover more than 10-30% and tree height of 2-5 m at maturity. Bamboos are considered to have evolved from prehistoric grasses between thirty and forty million years ago, long after the extinction of the dinosaurs. It then became the major food source for herbivorous animals, eventually becoming a food source for the modern human being as well. Bamboo originated in forests from an ancestral grass that closely resembled *Streptochaeta schrad* (Clark, 1996; Klinkenborg, 2001; https://www.bamboogrove.com). While bamboo grows everywhere in the world except those places with extremely cold climates, it is thought to have originated in China, where the first use of bamboo to make every day items was recorded (https://www.bamboogrove.com).

2.1.2 Taxonomy

Bamboos are perennial forest grasses classified under family Poaceae and subfamily Bambusoideae (Lobovikov *et al*., 2007; Han *et al*., 2009). Bambusoideaes are composed of 75 genera and approximately 1250 species of bamboos distributed in a range of environments from tropical and warm ecosystems to cold regions (Chaomao *et al*., 2006). The nearly 1,250 described species of bamboos are classified into three tribes: Arundinarieae (temperate woody bamboos, Bambuseae (tropical woody bamboos), and Olyreae (herbaceous bamboos) (Clark et al., 2015). Only two species of Bamboos, namely, highland bamboo /African alpine bamboo /

Mountain bamboo (*Arundinaria alpina* (K.Schum) and Lowland bamboo (*Oxytenanthera abyssinica (*A.Rich) Munro are found in Ethiopia.

2.1.3 Distribution and area coverage

Bamboos are distributed in a range of environments from tropical and warm ecosystems to cold regions. The majority of the bamboo species and their species richness are found in Asia, where with China having 626 species is the global leader in bamboo forest resource, followed by India: 102 species, Japan 84, Burma 75 and Malaysia 50. From Central and South America countries, Brazil 134, Venezuela 68 and Colombia 56 (Chaomao *et al*., 2006; Das *et al*., 2008). Madagascar is the leader in bamboo resources with 43 species in Africa and other African countries with potential bamboo resources include Tanzania, Malawi, Ethiopia, Uganda, and Zambia. Globally, 22 million hectares of land is thought to be covered by bamboos forest (ICBR, 2004 ; Toensmeier, 2016).

2.1.4 The biology of bamboos

Their high productivity, due to fast growth rates, and strong regeneration capabilities make them one of the most crucial forest resources in bamboo harboring regions of the world. The peculiar feature of bamboos is that their vegetative phase can last up to 100 years or longer without flowering and the plant life ends after flowering. Gregarious flowering pattern of woody bamboos followed by the subsequent death of the mother plant is a problem in a continuous supply of both economic and ecological benefits of bamboos (Sarma, 2009). On the basis of morphological habit, Bambusoideae are classified as woody and herbaceous bamboos (Ramanayake *et al*., 2007). Woody bamboos are known by their strongly lignified culms, specialized culm leaves, bisexual flowers, outer ligules on the foliage leaves, complex vegetative

branching and gregarious monocarpy (BPG, 2012). Herbaceous bamboos are known by shorter and more weakly lignified shoots, less vegetative branching, unisexual flowers, seasonal flowering pattern (BPG, 2012).

2.1.5 Reproduction and growth habit

The stem of Bamboo emerges from the ground at its full girth and grows to its full height in a single growing season (Xiaohong and Yulong, 2005). Morphologically, the bamboo shoot has a distal aerial part called the culm, a proximal ground level part called culm neck and a subterranean part called the rhizome i.e. underground part of the bamboo plant. Both culms and rhizomes consist of nodes and internodes but the rhizome nodes are responsible for bearing roots. Nodes consist of meristematic tissue from where culm sheath and a branch arise. The rhizomes collect and store the nutrients that sustain the life of the plant. Buds on the rhizomes develop into shoots that emerge from the ground to form a clump of culms. The speedy growth of culm is the reason why bamboo is considered as one of the fastest growing plants on earth (Brar, 2014). Bamboo produces four types of leave structures; these are heterophylls, foliar leaves, rhizome scales and culm sheath. Some of the bamboo species are evergreen while others are deciduous (Franklin, 2005). The main members of Bambusoideaes are arborescent and perennial woody species which grow up to height of 12 m and 30 cm in diameter in one growing season (Barker *et al*., 2001).

2.1.6 Economic uses

Moso bamboo (*Phyllostachys edulis)* forest covers an area of 3.87 million h, accounting for up to 70% of the Chinese bamboo forest area (Lobovikov *et al*., 2007; Song *et al*., 2011) . Because

of its rapid growth and highly lignified culms, the annual economic value of moso bamboo production, including timber and wood production, reaches 184 billion dollars (Li *et al*., 2016).

This tall, hearty grass was used for as many products as they could manage, as it was a quickly renewable resource. Bamboos have enormous economic utilizations. Widely used for pulp and paper production, housing, cattle fodder, spears, arrows, rafts, fishing rods, umbrellas, toys, beehives, as a roofing material, ladders, particle board, hand tools, brushes, pipes, sports goods, mats, scaffoldings, sticks, flooring, furniture, charcoal, fiber and textile, plywood for truck carriage and molding board for concrete (Qisheng *et al*., 2001; Liese, 2008). As highland bamboo is able to split easily, it is commonly used for making mats, chairs, sofas, tables etc. Bamboo is being recognized with its environmental services in general and climate change mitigation in particular (Ohrnberger, 1999).

2.1.7 Ploidy level / genome size and genetics

All previously reported chromosome counts of woody bamboos are tetraploid or hexaploid, whereas, the herbaceous bamboos are either diploids or tetraploids. Being mostly polyploid, bamboos are complex plants and genetical studies are very complicated. Moreover, natural populations are very heterogeneous. Most tropical bamboos are hexaploids ($2n=6x=72$) while most temperate bamboos are tetraploids ($2n = 4x= 48$) assuming a basic chromosome number of $x=12$ (Kellogg and Watson, 1993; Clark *et al*., 1995). It was later supported by flow cytometric estimation of the genomic DNA content (Gielis *et al*., 1997a; Gui *et al*., 2007).

Although several genomes have been sequenced or are being sequenced in the grasses family, we know little about the genome of the bambusoids (bamboos). The moso bamboo

(*Phyllostachys edulis)* genome size was estimated to be about 2034 Mbp by flow cytometry (FCM), using maize (cv. B73) and rice (cv. Nipponbare) as internal references (Gui et al., 2007).

Though genome and transcriptome sequences are available for several plants, there has been little effort and progress in the investigation of genomes of bamboos. The genetic information of many bamboo species is very poor except for Moso bamboo (*Phyllostachys edulis*), the most economical and widely distributed species in China (Paterson *et al*., 2005; Gui *et al*., 2007; Peng *et al*., 2013). Molecular genetics research is vital to promote genetic, evolutionary, functional studies of plants to have a comprehensive understanding of the biology and other key characteristics that enable to properly exploit plant resources in general and bamboo in particular (Das *et al*., 2008; Peng *et al*., 2013).

2.2 Bamboos in Ethiopia

2.2.1 Ethiopian lowland bamboo (*Oxytenanthera abyssinica* A.Rich Munro)

The genus *Oxytenanthera* belongs to the family Poaceae (Gramineae), subfamily Bambusoideae and tribe Bambusaceae (woody bamboos). The local name of this species in Affan Oromo is *Shimalla*, while in Amharic, it is called *Shimel*. Lowland bamboo is clump-forming monopodial bamboo with a strong rhizome up to 10 cm in diameter grows in pure stands. The culms/stems are grouped into large dense clumps erect and leaning with a length up to10 m (Kigomo and Kamiri, 1985). The base diameter varies from 3 - 5 cm. During shooting, culms are solid and later on develop into a small central cavity with a thick culm wall. Due to this, lowland bamboo is not easier to split for weaving as highland bamboo. The stripped leaves are about 20 cm long and during unfavorable environmental conditions, they have deciduous foliage (Liese, 2008).

One of the important features is that it is a hardy species on poor soils in dry vegetation formation. The species is very drought resistant, sustains itself with minimal rainfall and has very economical water uptake, as it tolerates rainfall down to 700 mm and a high temperature of up to 45°C (Kigomo and Kamiri, 1985). It helps maintain soil fertility by returning nutrients and humidity to the soil in the form of litter. Under conducive natural conditions, it grows at a density of 8000 stems/ha. The above-ground biomass is about 20 tonnes/ha (Embaye, 2003).

There is a morphological difference as some clumps have exceptionally thinner culms and others with much taller and thicker culms among the normal sized ones. Such differences could result from cross-fertilization when flowering. Lowland bamboo grows at an altitude range of 700 - 1,800 m above sea level. According to FAO and INBAR (2005), the major portion of Ethiopia's bamboo (85%) is the lowland bamboo found in the Combretum–Terminalia-Deciduous woodlands of Western Ethiopia together with other associated grasses. The major lowland bamboo growing areas of Ethiopia are Benishangul gumuz (Assosa, Kumruk, Pawi, Mandura, Dangur, Guba), Wollega (Gimbi), Gambella, Mytebri (Dereso, 2019). According to LUSO (1997) and Embaye (2003) the total area coverage of lowland bamboo in Ethiopia is estimated to be more than 800,000 ha, out of which only 481,000 ha was mapped and partially surveyed.

2.2.2 Ethiopian high land bamboo (*Arundinaria alpina* K.Schum)

The genus *Arundinaria* belongs to the family Poaceae (Gramineae), subfamily Bambusoideae and tribe Bambusaceae (woody bamboos). The vernacular names include, Mianzi /Mwanzi (Kiswahili), African alpine /mountain /highland bamboo (English), Babou Creux (French). This species is also known in different names in Ethiopian local languages. This includes, *Kerkeha* (Amharic), *Lemmen/ Shimela, (*Afan Oromo), *Anini (*Agew), *lema* (Konso*,* Kembata, Sodo

Gurage and Sidamo), *lewu* (Nuwer), *Kias (*Gamu), *Shenbek'wa* (Welayita), *Shineto /Shinato (*Kefigna); *werye /shikaro /Shinato* (Kefa) (Kelecha, 1987; IBC and GTZ, 2003; Bekele, 2007).

Highland bamboo is medium sized bamboo with straight culms of 12 - 20 m height and a base diameter of 8- 12 cm. The lanceolate leaves of about 7 cm length do not decompose easily in the cool climate and form often a thick soil cover of 10 cm and more. The occasional flowering, seeding and dying occurs in patches, no extensive flowering has been recorded (Bekele, 2007; Liese, 2008).

The highland bamboo grows in the highlands of East Africa at a lower temperature between 10-20 ^{o}C at an altitude of between 2200 -3500 m. Highland bamboo is found in highlands and humid parts of Ethiopia having a short dry season and rainfall between 1500 - 2500 mm. According to FAO and INBAR (2005) the area of forest covered by highland bamboo in Ethiopia is mapped naturally grown is 129,626 ha and the area planted by farmers is estimated to be about 19,000 ha, together, totaling up to 148,626 ha.

Areas of Ethiopia with abundant highland bamboo resources includes, Jima (Agaro, Gera), Gore, Bore,Gujji, HagereSelam, Western Shewa (Ambo,Tikure-Inchini, Shenen, Jibat Mountain), Bale (Harena forest and Shedem Kebele), Western Aris (Degaga, Munesa, Shashemene) in Oromiya regional state, Awi-zone/Inijibara, Gojam/Choke mountains, South Wello (Denkoro forest and) and South Gonder (Debere Tabor), Debresina/Wofwasha and Ankober in Amhara regional state and Sidama, Bonga (Ameya, Wushwush), Bench-Maji, Kefecho Shekech (Ameya, Baha-Chapa, Gada, Gecha-Masha, Andracha, Chencha/Arbaminch, Mizan Teferi/Kulish, Dawaro) and Gurage /Indibir-Jembero in Southern Nations, Nationalities and Peoples Regional State (Desalegn and Tadesse , 2014)

The rhizomes of highland bamboo exhibit a monopodial branching pattern which gives the plant a clump-forming habit. The clumping habit is evident especially in plants under cultivation. The species, however, has elongated rhizome necks, and the culms are usually widely spaced, forming a loose or open clump. In dense natural stands, it becomes difficult to distinguish one clump from another. The culm of highland bamboo is hollow but it typically has a thick wall, which makes it ideal for the production of strong panel products (Bekele, 2007; Liese, 2008).

2.3. RNA Sequencing (RNA-Seq)

RNA-Sequencing (RNA-Seq) is referred to as rapid analysis of plant's fluctuating transcriptome through sequencing cDNA that allows sequencing of diverse RNA transcripts in a cell including mRNA transcripts, miRNA, tRNA and small RNA (Duhoux *et al*., 2015). Rapidly evolving innovations, decreasing sequencing cost and time is transforming plant sciences research. Plant researchers use state of the art sequencing tools to assist plant improvement by understanding plant biochemistry and physiology. Without the need of protein purification and characterization, characterization of a cellular RNA transcript is a gateway to get at hard to do plant biochemistry (Martin *et al*., 2013 ; Kellner *et al*., 2015).

The technologies and tools for RNA-Seq and transcriptome discovery are advancing at a fascinating pace (Martin *et al*., 2013). Further and deeper investigation of genes having altered and erratic expression is very important as it supports complex traits such as abiotic stress. Investigation in to the genome-wide differential RNA expression under diverse environmental conditions may divulge insights in to biological corridors, complexity and molecular

mechanisms that control the role of genes take part in abiotic stress tolerances and adaptation (Vanverk *et al*., 2013; Voytas, 2013; Bhardwaj *et al*., 2015; Dolgin, 2015).

During the occurrence of abiotic stress, RNA-Seq is used to detect differentially expressed genes and possibly identify changes in cellular pathways. RNA-Seq has enabled to easily know the affiliation between genes and their corresponding traits which allows isolation of genes for traits whose biochemistry is difficult to decipher (Kellner *et al*., 2015; Góngora-Castillo *et al*., 2012). So RNA-Seq is capable of illuminating structural and regulatory networks to uncover how plants respond to fast-evolving circumstances and their environments (González-Ballester *et al*., 2010). Through RNA-Seq analysis, it is possible to score the existences of unique RNA existence and quantity from novel genomes of hardy plants well adapted to abiotic stress at a specific point of plant developmental and physiological stage. Analysis of genome-wide differential RNA expression entails researchers with promising insights in to molecular mechanisms and biological pathways that regulate development, cell fate and changes due to and during abiotic stresses (Duhoux *et al*., 2015).

By using various bioinformatics tools, analysis of RNA-Seq sequences enables to look at posttranscriptional modifications; alternative gene spliced transcripts, transcription start sites, exon/intron boundaries, new splicing variants, gene fusion, gene boundaries and investigates the differences in the level of gene expression. One of the most important features of RNA-Seq is that it does not discriminate against low-abundance transcripts and enriched transcript termini (Nagalakshmi *et al*., 2010).

2.4 Transcriptomics

A transcriptome is a complete set of transcripts in a cell, both in relations of type and quantity. The study of transcriptomics has eased due to the advent of high-throughput sequencing-based methods. The starting of direct sequencing of complementary DNAs (cDNAs) using high-throughput DNA sequencing technology has transformed transcriptomics studies (Severin, 2010). Transcriptome assembly study is credited as it diminishes the intricacies of ploidy, entire or partial genome duplication, paralogy, heterozygosity and genome sizes with repetitive sequences. It lets access to the gene space of un-sequenced recalcitrant species. The advancement of technological precision in sequencing platforms, software and elegant algorithms is expected to improve management and mining of large, complex, repetitive plant genomes. This enables to have better knowledge of plants' physiology and biochemistry that can be easily applied for improving plants for economic traits of interest (Moerkercke *et al*., 2013).

Through transcriptome studies of various plant species many of the common signaling pathways are functional in xerophytes adopted to arid and semi-arid environmental conditions (Farrant et al., 2015; Costa et al., 2016, 2017). The big argument pertaining the suitability of stress sensitive model species like Arabidopsis vs. xerophytes/desiccation/ resurrection plants in pursuit to study abiotic stress response remains unresolved. The decisive question remains how studies on resurrection plants are going to lead us to novel genes and stress signaling pathways, when so far, many transcriptome studies have mostly delivered on general stress pathways?

Searching for novel genes in resurrection plants is very important than relying on model plant species. Interestingly, transcriptome studies of resurrection plants not only found a high proportion of unknown transcripts 33% *Craterostigma plantagineum* (Rodriguez et al., 2010) and ~40% *Haberlea rhodopensis* (Gechev *et al*., 2013), but in the case of the *Craterostigma plantagineum* transcriptome, also identified many taxonomically restricted genes (TRGs) and non-protein coding RNAs (ncRNAs) (Giarola *et al*., 2014; Giarola and Bartels, 2015). TRGs are known to code for new traits required for the adaptation of organisms to particular environmental conditions (Johnson and Tsutsui, 2011), and it has been suggested that these may harbor the potential for novel gene discovery linked to desiccation tolerance.

2.4.1 Bamboo transcriptome studies

Profiling gene expression through transcriptome analysis in a given tissue or under an experimental condition, such as responses to environmental stresses, is a fundamental prerequisite to understanding how functional networks operate in living system of a given plant. Such analysis helps to decipher the genome-wide quantification of gene expression, detection of novel transcripts, identification of alternatively spliced genes and detection of allele specific expression for the plants species under investigation (Moustafa and Cross, 2016) .

In light of this transcriptome studies of bamboos under abiotic stress conditions have been conducted. These include, transcriptome and comparative gene expression analysis of *Phyllostachys edulis* in response to high light (Zhoa *et al*., 2016), genome-wide identification and characterization of TIFY family genes in Moso Bamboo (*Phyllostachys edulis*) and expression profiling analysis under dehydration and cold stresses (Huang *et al*., 2016), genome-

wide analysis and expression profiling of the Heat Shock Factor gene family in *Phyllostachys edulis* during development and in response to abiotic stresses (Xie *et al*., 2019).

Transcriptomics studies on different species of bamboos on non stress aspects include transcriptome sequencing and analysis of the fast growing shoots of Moso Bamboo (*Phyllostachys edulis*)(Peng *et al*., 2013), characterization of the floral transcriptome of Moso Bamboo (*Phyllostachys edulis*) at different flowering development stages by transcriptome sequencing and RNA-Seq Analysis (Gao *et al*., 2014), comparative transcriptome analysis reveals hormone signaling genes involved in the launch of culm-shape differentiation in *Dendrocalamus sinicus* (Chen *et al*., 2017), hormone distribution and transcriptome profiles in bamboo shoots provide insights on bamboo stem emergence and growth (Gamuyao *et al*., 2017) and phenotypic and comparative transcriptome analysis of different ploidy plants in *Dendrocalamus latiflorus* Munro (Qiao *et al*., 2017),, transcriptome analysis provides insights into xylogenesis formation in Moso bamboo (*Phyllostachys edulis*) shoot (Zhang *et al*., 2018),

2.5 Plant abiotic stress

During their entire life span plants are exposed to different environmental conditions. Due to their sessile nature plants have to develop their own mechanism to cope up the changing environmental conditions, such as exposure to drought, cold, salinity, and extreme temperature which may adversely affect plant growth, development and hence their productivity (Agarwal *et al*., 2012; Akhtar *et al*., 2012). When plants face serious stresses, a series of genes with diverse functions are either repressed or enhanced (Lata *et al*., 2011). Such proteins with the significant role during the occurrence of stresses are functional and regulatory proteins.

Among the various abiotic factors challenging plant growth and development globally, drought and salt stress are the most important ones. Drought is a meteorological term and is usually defined as a combined interplay of reduced rainfall, decreasing groundwater table, limiting water availability with the rise in temperature (Singh and Laxmi, 2015; Singh *et al*., 2015). When exposed to drought, plants encounter multiple impediment including, cell injury through reactive oxygen species (ROS), generation and increasing cellular temperature, which ends up in an increase in the viscosity of cellular contents, alterations in the protein-protein interactions and protein aggregation and denaturation (Farooq *et al*., 2008). Due to dehydration, cell shrinkage followed by a noticeable decline in cellular volume becomes an evident phenomenon. Higher accumulation of solutes causes toxicity and negatively affects the functioning of some enzymes, often leading to diminished photosynthesis and water use efficiency (WUE). Under longer exposure to dehydration, plants show leaf rolling followed by wilting and bleaching that finally ends up in the death of the plant (Sahoo *et al*., 2013). Plant reproductive stages, flowering and seed development are particularly susceptible to drought stress (Alqudah *et al*., 2010; Samarah and Alqudah, 2011).

Salt stress in plants is a condition where excessive salts in the soil solution cause inhibition of plant growth or plant death. On a global scale, no toxic substance restricts plant growth more than does salt (Zhu, 2007). The increment of $Na+$ and Cl^- ions in the soil causes many physiological disorders in plants (Tavakkoli, *et al*., 2010). The physiologically toxic level of salinity induces nutrient unavailability, altered levels of growth regulators, enzymatic inhibition, membrane damage and metabolic malfunctioning including photosynthesis which eventually

result into demise of the plant (Turkana and Demiral, 2009; Ali *et al*., 2012). As a result of such problems, it is very important to exert all possible efforts in developing plants that can be productive within the face of saline environmental conditions.

The effects of toxicity caused by high salinity levels in plants may be divided into two mechanisms: (1) disturbance in osmotic regulation and (2) ionic toxicity. Osmotic disturbance caused by salinity stress causes dehydration which leads to the inhibition of water uptake, cell elongation, and development of leaves(Gupta and Huang, 2014). The second phase, ionic stress leads to the excess rise of sodium ions while potassium ions significantly decrease. Such nutrient imbalance leads to toxicity which results in the hasty aging and dying of leaves, and impediment of enzyme activity, protein synthesis and photosynthetic ability (Munns and Tester, 2008) .

During the early stage of osmotic and ionic stress, signal transduction may take place which may either establish osmotic adjustment and ion homeostasis or cell death. In the event that the signal transduction successfully initiates osmotic adjustment and ion homeostasis, recovery or adaptation of the plant is highly expected. However, if cell death occurs, then the chances that the plant recovers from the salinity-induced toxicity become significantly low (Munns and Tester, 2008)

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During the initial stages of high salinity stress, various physiological changes can be observed in plants which include interruption of membranes, nutrient imbalance, impaired ability to detoxify reactive oxygen species (ROS), alteration of antioxidant enzymes, reduced and

altered photosynthetic activity, and decreased stomatal aperture (Sharma and Dubey, 2005; Tanou *et al*., 2009). Interruption of cell membranes is largely attributed to excessive accumulation of sodium in the cell walls which leads to osmotic stress and even cell death. Nutrient imbalance, on the other hand, takes place since salts in the soil serve as important sources of nutrients for the plants (Shrivastava and Kumar, 2015). Trouble in nutrient balance, such as in the case of high salinity levels, the nutrients available for plants to absorb also become imbalanced (Shrivastava and Kumar, 2015). Impaired photosynthetic ability associated with high salinity stress is due to the decrease in leaf area, chlorophyll content and stomatal conductance, and reduced photosystem II efficiency caused by high salt levels (Sharma *et al*., 2012).

The main mechanism used by plants to recover from the osmotic stress induced by high salinity levels is through the osmolytes and osmoprotectants (Hasegawa *et al*., 2000; Munns and Tester, 2008). Sugars, cyclic and acyclic polyols, amino acids and derivatives of amino acids, fructans, quaternary amino and sulfonium compounds are some of the known organic solutes capable of accumulating in the cells of plants to regulate osmosis during stress (Munns and Tester, 2008). The compounds mentioned above are collectively known as compatible solutes or osmolytes due to their ability to accumulate in excessive amounts without impairing cellular functions (Cushman, 2001). Compatible osmolytes form massive units of compounds that function in restoring the osmotic potential of cytoplasm to facilitate water uptake and maintain turgor in the cell (Cushman, 2001).

Naturally, plants use diverse mechanisms to deal with salt stress; the most important strategy is minimizing the amount of salt taken by the roots and partitioning to the tissue and cellular levels

so as to avoid the accumulation of toxic concentrations in the cytosol of functional leaves (Ali *et al*., 2012). Other mechanisms used by plants to battle salt stress includes, maintenance of reduced osmotic potential through synthesis and accumulation of compatible solutes, maintenance of photosynthesis, vacuolar sequestration of toxic Na+ (23) ion exclusion at root level and ion secretion in leaves through salt glands (Bradley and Morris, 1991; Bedre *et al*., 2016), osmotic adjustment, ion homestasis, growth regulation and redox equilibrium (Goyal *et al*., 2016) .

The expression of diverse salt-response genes plays a decisive role in the attainment of the combating mechanisms through facilitating biochemical and physiological changes. These involve groups of structural protein-coding genes, including late embryogenesis abundant (LEA) proteins, antioxidant proteins, osomo-regulatory genes, anti-porter/transporters, signal-related protein kinases and most importantly active involvement of transcription factors like, bZIP, NAC, MYB, WRKY and ERF (Ali *et al*., 2012) . The interaction of the above genes and proteins is the basis for the formation of a number of pathways that makes plants capable of withstanding the effect of salinity on their growth and development. Some of the pathways include, the mitogen-activated protein kinase (MAPK) pathways, salt overly sensitive (SOS) pathway and calcium-dependent protein kinase (CDPK) pathway (Zhu, 2003; Ludwig *et al*., 2004; Nakagami *et al*., 2005; Goyal *et al*., 2016). Besides such pathways, a couple of plants hormones, like salicylic acid, jasmonic acid and abscisic acid also contribute an important role in stress signaling and adaptation (Xiong and Zhu, 2002; Fujita *et al*., 2006; Ma *et al*., 2006). Despite the promising progress made in investigating the processes of molecular mechanisms behind salt stress tolerances, the intricacy of interactions involved in the salt stress tolerance mechanisms in plants is demanding further more in-depth analysis. For this reason, there is a call

for developing a clear intellectual capacity of salt stress mechanisms of plants and identify the most important mechanisms involved in directing salt tolerance (Ma *et al*., 2006).

2.6 Plant transcription factors

Plant transcription factors (TFs) are regulatory proteins which regulate gene expression in response to various stresses including drought and salinity. Thus TFs play a significant role in plant growth and development by regulating defense response and gene regulation networks (Feng *et al*., 2017). TFs contain a DNA binding domain which binds to cis-acting elements which are present in the upstream region of all gene promoters (Loredana *et al*., 2011). On the basis of their protein products, plant genes induced due to abiotic stress are divided into two main groups. The first types of genes are regulatory proteins which operate in the signal transduction networks, this includes transcription factors, molecular chaperones, functional proteins (Song *et al*., 2013). The second types of genes are those coding for products that directly let cells to resist environmental stresses, these include malondialdehyde (MDA), late embryogenesis abundant (LEA) protein, betamine and other anti- freezing proteins and osmotic regulators (Loredana *et al*., 2011).

A network of plant transcription factors with their binding sites directly controls transcription of a plant gene (Chaves and Oliveira, 2004). TFs control gene regulation due to their role in repressing or activating the activity of RNA polymerase. Nearly 10% of genes in the plant genome encodes for transcription factors (Franco-Zorrilla *et al*., 2014). On the basis of their DNA binding domain, TFs are classified into various families (Riechmann *et al*., 2000). The most important ones, particularly associated with abiotic stress are, MYB, NAC, AP2/ERF,

bZIP, bHLH, WRKY and others (Khan *et al*., 2018). As abiotic stresses are quantitative traits, it might require the regulation of numerous genes including the TFs and in most cases, single TFs may regulate multiple genes which are actively involved in abiotic stress responses. Conducting a compressive study on all TFs associated with abiotic stress regulatory mechanisms is very important particularly in economic species whose growth and productivity is badly impeded due to abiotic stress (Kimotho *et al*., 2019). The graphical representation of transcriptional regulatory networks of transcription factors is shown in Figure one (Wang *et al*., 2016).

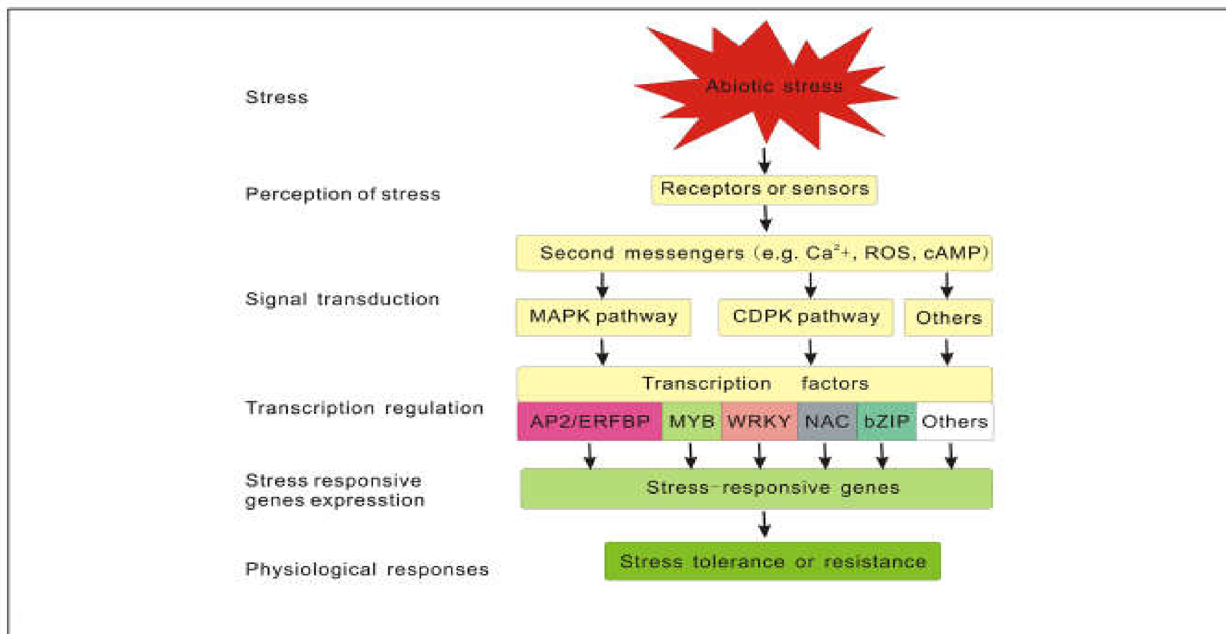

Figure 1. Generic signaling pathway involved in plant abiotic response

2.6.1 Basic leucine zipper (bZIP) transcription factor gene family

Transcription factors (TFs) consist of one or more sequences-specific DNA binding domains that regulate gene expression through binding to the promoter and/or enhancer regions of target genes. Specific transcription factors (TFs) are highly responsible for the expression of proteins

with diverse functions. The basic leucine zipper (bZIP) transcription factor family is one of the largest and most conserved plant TFs (Khan *et al*., 2018). bZIP is 60-80 amino acids in length and comprised of two parts; a leu zipper and a basic region. These two parts are both functionally and structurally distinct. The Leu zipper is less conserved dimerization motif and comprised of heptad repeats of Leu or other bulky hydrophobic amino acids located just nine amino acids towards the C-terminus and mediates homo- and/or hetro-dimerization of bZIP proteins. The basic region composes about 16 amino acid residues with the invariant motif N-x7-R/K-x9 is highly conserved and responsible for nuclear localization and DNA binding (Ptashne and Gann, 1997; Zhang *et al*., 2017).

Some of the transcription factors like NAC are found only in plants. But bZIP TFs are found in many eukaryotic genomes including humans. Members of numerous bZIP TFs have been identified in many eukaryotic genomes. A total of 15, 498 bZIP TFs have been identified from various genomes (Jin *et al*., 2017). The number of identified bZIP TFs ranges from 17 in *Saccharomyces cerevisiae* (Fassler *et al*., 2002) to 200 in hexaploid hulless oat (Wu *et al*., 2017). Others include 49 in Castor bean (Jin *et al*., 2014), 55 in grapevine (Liu *et al*., 2014), 56 in human (Vinson *et al*., 2002), 75 in *Arabidopsis* (Jakoby *et al*., 2002), 89 in rice (Nijhawan *et al*., 2008), 92 in *Sorghum* (Vanitha and Ramachandran, 2011), 125 in maize (Wei *et al*., 2012), 131 in Soya bean (Liao *et al*., 2008), 162 in lowland bamboo (Adem *et al*., 2019).

The genome-wide survey and characterization of bZIP TFs have been conducted for many plant species including non-model plants (Wang *et al*., 2011; Wang *et al*., 2017). This include in

Arabidopsis (Jakoby *et al*., 2002), maize (Wei *et al*., 2012), rice (Nijhawan *et al*., 2008), sorghum (Wang *et al*., 2011), cucumber (Baloglu 2014), grapevine (Liu *et al*., 2014) and barley (Pourabed *et al*., 2015) with the availability of bZIP whole genome sequences for each plant species.

bZIPs transcription factors are involved in various plant physiological and developmental processes as well as biotic/abiotic stress responses. So, bZIP TFs play a significant role in assisting plants to withstand unfavorable environmental conditions (Wang *et al*., 2011; Wang *et al*., 2018). The vital role of bZIP TFs in developmental processes have been demonstrated in cell elongation (Fukazawa *et al*., 2000; Liu *et al*., 2015), organ and tissue differentiation (Silveira *et al*., 2007; Shen *et al*., 2007), somatic embryogenesis (Guan *et al*., 2007) seed storage protein regulation (Lara *et al*., 2003), carbon / nitrogen and energy metabolism (Weltmeier *et al*., 2006 ; Baena-Gonzalez *et al*., 2007), unfolded protein response (Liu *et al*., 2007), ripening (Hu *et al*., 2016). bZIP TFs crucial role as active regulators to various abiotic/biotic stresses like high salinity, drought, cold stress and disease resistance have been confirmed in many plants including, maize (Kusano *et al*., 1995), *Arabidopsis* (Weltmeier *et al*., 2009; Yang *et al*., 2009), barley (Xue *et al*., 2004), rice (Shimizu *et al*., 2005; Mukherjee *et al*., 2006; Xiang *et al*., 2008), wheat (Kobayashi *et al*., 2008), *Chlamydomonas reinhardtii* (Ji *et al*., 2018), bacterial blight resistance in cassava (Li *et al*., 2017). The study of bZIP TFs in green plant evolution suggested that the ancestor of green plants possessed four bZIP genes functionally involved in oxidative stress, unfolded protein response and light-dependent regulations (Corrêa *et al*., 2008). Four transcript factors including bZIP were found to have a positive influence in cadmium tolerance in creeping bentgrass (Yuan *et al*., 2018).

2.7. Gene Ontology (GO), Clusters of Orthologous Groups of proteins (COGs) and Kyoto Encyclopedia of Genes and Genomes (KEGG)

Gene Ontology (GO) is an internationally standardized gene function classification system that provides a dynamically updated controlled vocabulary to fully describe gene and gene products in organisms (Li *et al.,* 2012). Significant enrichment analysis of GO function can determine the major biological functions of differentially expressed genes. GO analysis is very important for annotation and comparing of gene products of different species to gain general insights into the biological roles of all genes revealed by high-throughput analysis. The output of the analysis is typically a ranked list of GO terms, each associated with a p-value (Rhee *et al*., 2008).

The database of Clusters of Orthologous Groups of proteins (COGs) is a tool for genome-scale analysis of protein functions and evolution. The current COG database contains both prokaryotic clusters (COGs) and eukaryotic clusters (KOGs). The COG database is accompanied by the cognitor program that is used to fit new proteins into the COGs and can be applied to the functional and phylogenetic annotation of newly sequenced genomes (Roman *et al*., 2000).

The metabolic pathways of unigenes are investigated using Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation system. This database systematically analyzes gene function and integrates genomics, biochemistry and systems functional information and draws corresponding information on different types of biological processes (Kanehisa *et al*., 2008). Annotating genes on the KEGG Bio-Pathway database, allows seeing which bio-pathways these genes are involved in and which important biological functions are shown (Ge *et al*., 2014). The KEGG database which deals with genomes, biological pathways, and chemical substances was used to investigate

the gene product during plant metabolism and associated gene functions in cellular processes (Kanehisa and Goto, 2000).

KEGG aims to link genomic- and molecular-level information to higher-level functions of the cell, organism and the ecosystem. Annotation with KEGG is based on associating molecular function with orthologous groups, which are defined based on clustering of genes from completed genomes (currently, >4000 genomes), using the KEGG's internal 'KEGG Orthology and Links Annotation' (KOALA) program (Kanehisa *et al*., 2016). Any functional annotation derived by simple sequence similarity transfer should be scrutinized carefully before embarking on a particular hypothesis about this particular protein. Absences of annotation does not mean absences of function. Furthermore, absence of a specific annotated gene in a plant genome/transcriptome does not necessarily mean that the plant cannot perform a particular function. Functional annotation is highly dependent on complete gene models, so in cases of partial or incomplete gene models, as is frequently seen with transcriptome assemblies, the tools used might not be sensitive enough to ascribe (the correct) function on a partially assembled gene (Bolger *et al*., 2017).

2.8 Molecular markers

Molecular markers, a fragment of DNA which is associated with a particular region of the genome, have played a significant role in the study of genetic diversity, genetic variability, genotype identification, plant breeding and population genetics (Varshney *et al*., 2007; Jiang *et al*., 2013). In bamboos where traditional methods of genotype identification are difficult and time- consuming, molecular markers provide alternative tools which are not only precise and time-saving, but also address various aspects of bamboo taxonomy. Considerable progress has been achieved in bamboos using molecular markers ranging from species identification, studying genetic diversity, testing clonal fidelity and establishing phylogenetic relationships (Das *et al*.,

2005; Yang *et al*., 2012). Higher level phylogenetic relationships within the bamboos of sub-family *Bambusoideae* was established based on five plastid DNA that revealed three to five major lineages showing distinct bio-geographic distribution (Sungkaew *et al*., 2009).

There is no single best molecular marker that meets the entire requirements. All markers have their own merits and demerits. But an ideal molecular marker is expected to have the following characters.1) highly polymorphic nature: It must be polymorphic as it is polymorphism that is measured for genetic diversity studies. 2) Co-dominant inheritance: determination of homozygous and heterozygous states of diploid organisms. 3) A frequent occurrence in the genome: A marker should be evenly and frequently distributed throughout the genome. 4) Selective neutral behaviors: the DNA sequences of any organism are neutral to environmental conditions or management practices. 5) Easy access (availability): It should be easy, fast and cheap to detect. 6) High reproducibility. 7) Easy exchange of data between laboratories (Kumar *et al*., 2009). Molecular marker technology has constantly progressed from hybridization-based Restriction fragment length polymorphism (RFLP) to Polymerase chain reaction (PCR)-based Randomly amplified polymorphic DNA (RAPD), Amplified fragment length polymorphism (AFLP), Inter-Simple sequence repeat (ISSR) and Simple sequence repeats (SSR), high-throughput Single nucleotide polymorphism (SNP) and more lately, ultra-high-throughput genotyping by sequencing (GBS) has been established (Mir *et al*., 2013).

2.8.1 Microsatellite (SSR) markers

SSR markers, as one of the powerful markers in plant biology refers to DNA sequences of 1-6 bp in length that are tandem repeated a variable number of times (Jurka and Pethiyagoda, 1995). When compared with other markers, SSR markers provides many desirable features including,

PCR screening, hyper-variability, multi-allelic nature, co-dominant inheritance, reproducibility, relative abundance and extensive genome coverage, followed by denaturing gel electrophoresis for allele size determination (Kumar *et al*., 2009, Manoj *et al*., 2013). Microsatellites are also fluorescent-labelled and detected (Schuelke, 2000). As a result SSR were considered to be reliable for construction of QTL mapping (Jeennor and Volaert, 2014), high-density linkage maps (Sugita *et al*., 2013), cultivar identification (Ercisli, *et al*., 2011), genetic diversity analysis (Moretzsohn, *et al*., 2004) , marker-assisted selection (Sui *et al*., 2009).

CHAPTER 3

3. RNA-Seq global transcriptome profiling of Ethiopian lowland bamboo (*O.abyssinica*) under drought and salt stress

3.1 Materials and Methods

3.1. 1 Plant materials, growth conditions and treatment

Lowland bamboo seeds used in this research were obtained from Ethiopian Biodiversity Institute collected from Asossa area, West Ethiopia. Germination and growth was conducted under plastic pots with room conditions of 26 °C/22 °C (day/night), with 75% relative humidity, 16 hr photoperiod and 175 µmol/ (m^2 .s^{-1}) light intensity (**Appendix 1**). One month old seedlings, with height range of 30 cm to 35 cm , were transferred to water media for one week to create similar condition before treatment (**Appendix 2**). A total of 90 seedlings (30 seedlings per treatment, 10 seedlings per replication) were used for control, salt and drought stress with three biological replications. The seedlings were treated with 200 mM NaCl and 25% PEG-6000 (Poly Ethyleneglycol) to induce salt and drought stress, respectively. The three biological replicates for 24 hrs treatment were named as W1, W2, W3 for control, N1, N2, N3 for salt and P1, P2, P3 for drought, while the 48 hrs treatment were W4, W5, W6 for control and N4, N5, N6 for salt. Seedlings treated with 25% PEG-6000 wilted and could not be used for RNA extraction at 48hrs. After treatment, 15 independent leave samples (six control, six salt and three drought stresses) were collected and immediately frozen by liquid nitrogen and stored at -80° C until use. Samples for RNA extraction were taken from seedlings of each flask that were thought to have good RNA. The samples taken from each flask were bulked as one sample.

3.1.2 RNA extraction, cDNA synthesis and sequencing

Total RNA of each sample was extracted using TRIzol Reagent (Invitrogen) kit. Quantity and quality of total RNA of each sample was checked by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), Nano Drop (Thermo Fisher Scientific Inc.) and 1% agarose gel. One µg total RNA with RIN value above 7 which has a good integrity (Schroeder *et al*., 2006) was used for following cDNA synthesis and library preparation. The poly (A) mRNA isolation was performed using NEBNext Poly (A) mRNA Magnetic Isolation Module (NEB). The mRNA fragmentation and priming was performed using NEBNext First Strand Synthesis Reaction Buffer and NEBNext Random Primers. First strand cDNA was synthesized using ProtoScript II Reverse Transcriptase and the second-strand cDNA was synthesized using Second Strand Synthesis Enzyme Mix. The purified double-stranded cDNA by AxyPrep Mag PCR Clean-up (Axygen) was then treated with End Prep Enzyme Mix to repair both ends and add a dA-tailing in one reaction, followed by a T-A ligation to add adaptors to both ends. Size selection of Adaptor-ligated DNA was then performed using AxyPrep Mag PCR Clean-up (Axygen), and fragments of ~360 bp (with the approximate insert size of 300 bp) were recovered. Each sample was then amplified by PCR for 11 cycles using P5 and P7 primers, with both primers carrying sequences which can anneal with flow cell to perform bridge PCR and P7 primer carrying a six-base index allowing for multiplexing. The PCR products were cleaned up using AxyPrep Mag PCR Clean-up (Axygen), validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and quantified by Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Then libraries with different indices were multiplexed and loaded on an Illumina HiSeq instrument according to manufacturer's instructions (Illumina, San

Diego, CA, USA). Sequencing was carried out using a 2x150 bp paired-end (PE) configuration; image analysis and base calling were conducted by the HiSeq Control Software (HCS) + OLB + GAPipeline-1.6 (Illumina) on the HiSeq instrument.

3.1.3 Data filtering and assembly

Quality assessment of the raw paired-end reads was performed using FastQC v0.11.2 (Andrews, 2010). Pre-processing of raw reads was conducted using Cutadapt v.1.9.1 (Martin and Marcel, 2011) for adaptor trimming and Sickle v1.33 (Joshi *et al*., 2011) for quality filtering. Clean reads were mapped using Bowtie 2 software. The aligned reads were then used for transcriptome assembly using Trinity v2.2.0 (Grabherr *et al*., 2011). Trinity works by integrating three different software modules: Inchworm, Chrysalis, and Butterfly, execute one after the other to process bulky volumes of RNA-seq reads into full-length transcripts. The duplicated contigs were removed using CD-HIT v4.5.4 (Fu *et al*., 2012). The longest transcripts were taken to be unigenes for functional annotation by identifying nucleotide sequences of all transcripts.

3.1.4 Functional annotation and classification of de novo assembled unigenes

The *de novo* assembled lowland bamboo unigenes were annotated through homology searches against public protein databases with an (E-value cut-off of 10^{-5}) (Zhao *et al*., 2016). The unigene sequences were annotated by using Blast2GO (Gotz *et al*., 2008). The databases used for annotation includes NCBI-Nr, COG, Swissprot, KEGG and GO. GO-Term Finder was used for identifying Gene Ontology (GO) terms that annotate a list of enriched genes with a significant *P*- value < 0.05.

3.1.5 Gene expression and differentially expressed genes (DEGs) analysis

For expression analysis the unigene sequence file as a reference gene file, RSEM v1.2.6 (Li and Dewey , 2011) was employed to estimate gene and isoform expression levels from the pair-end clean data. FPKM (Fragment Per Kilobases per Million reads) was calculated and employed to quantify expression abundance of transcripts in each sample. For differential expression analysis, the DESeq2 v1.6.3 (Anders and Huber, 2012) R program, a model based on the negative binomial distribution was used for determining differential expression from digital gene expression data. A DESeq2 analysis was performed using three combinations: (i) Control vs. drought, (ii) control vs. salt and (iii) drought vs. salt. To control false discovery rate, *P*- value < 0.01 was adjusted by Benjamini and Yekutieli's (2001) approach. Then, genes with $|\log_2$ fold change$| > 1$ for (up or down) and adjusted *P*- value < 0.05 were treated as differentially expressed (Zhao *et al*., 2016).

3.1.6 Validation of RNA-Seq data by RT-qPCR

To validate RNA-Seq data by qRT-PCR 40 candidate unigenes (20 up-regulated and 20 down-regulated) were randomly selected. After removing conserved regions using Expasy (http://expasy.org/) from the protein sequences, 48 qPCR primers (**Appendix 3**) were designed and synthesized from the full length cDNA sequences of each unigene using Primer quest tool. The RT-qPCR was performed on an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using the SYBR Premix Ex Taq™ kit (Takara, Dalian, China), according to the manufacturer's instructions. The PCR amplification comprised a 5 min incubation at 94 °C then a cycle of 94 °C for 30 s, 60 °C for 30s, 72 °C for 1 min, repeat 35 times, 72 °C for 10 min, and a final 4 °C hold. Relative expression of qRT-PCR was calculated using $\mathbf{2^{-\Delta\Delta CT}}$. NTB-F

TCTTGTTTGACACCGAAGAGGAG and NTB-R AATAGCTGTCCCTGGAGGAGTTT primer was used from a gene that was confirmed to be used as references for qRT-PCR from Chinese Moso bamboo (Fan *et al*., 2013).

3.2 Results

3.2.1 Sequencing, *de novo* assembly and functional annotation of lowland bamboo transcriptome

The result of Illumina paired-end sequencing and *de novo* assembly of lowland bamboo is presented in **Table 1**. and length distribution of 406,201 unigenes is illustrated in **Figure 2**.

Table 1 Summary of Illumina paired-end sequencing and de novo assembly of lowland bamboo.

Reads/contigs/unigenes	Read/contig/unigenes Parameters	Value /number
Sequenced reads	Number of raw reads	809,219,680
	Number of clean reads	754,444,646
	Total read length (bp)	99,601,349,229
	GC content	53.72 %
	Q20 percentage	97.4 %
	Q30 percentage	93.09 %
Contigs	Total number	9,595,574
	Total length(bp)	1,101,480,760
	Mean length (bp)	374
	Contig N50 (bp)	566
Unigenes/transcripts	Total number	406,201
	Total length (bp)	240,231,095
	Mean Length(bp)	641
	Unigene N50(bp)	873
	Minimum length(bp)	201
	Maximum length(bp)	16651

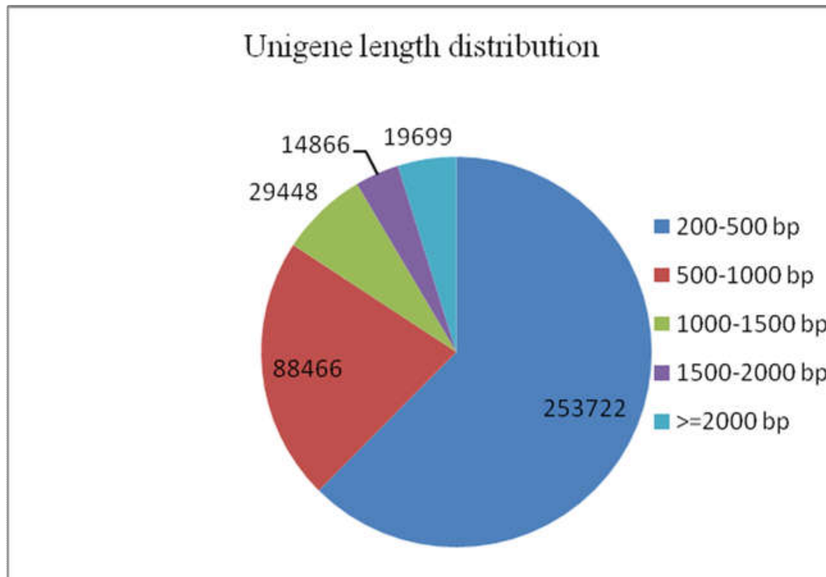

Figure 2. Unigene length distribution

As there was no genomic and transcriptome information published so far on lowland bamboo, NCBI-Nr blast search of the unigenes was conducted against other species. Accordingly, *Oryza sativa japonica* was identified to be the species with the highest homology (41,400 unigenes, 10.2%), followed by *Brachypodium distachyon* (25,886) and *Setaria italica* (21, 881). Other species with considerable matches include *Oryza brachyantha* (18,081), *Zea mays* (15,194), *Sorghum bicolor* (12, 697), *Oryza sativa indica* (9, 504), *Aegilops tauschii* (8,162), *Triticum urartu* (7,393) and *Phyllostachys edulis* (6, 395).

From the total 406,181 identified unigenes 217,067 (53.4%) were successfully annotated using BLASTX searches against the public databases of NCBI-Nr (203,777, 93.8%), Swissprot (115,741, 53.3%), COG (81,632, 37.6%) and KEGG (80,587, 37%). But, the remaining 189,114 (46.6 %) unigenes did not show any significant functional similarity to any of the database

explored. The detailed distribution of annotated unigenes to each and multiple databases is shown in **Figure 3**.

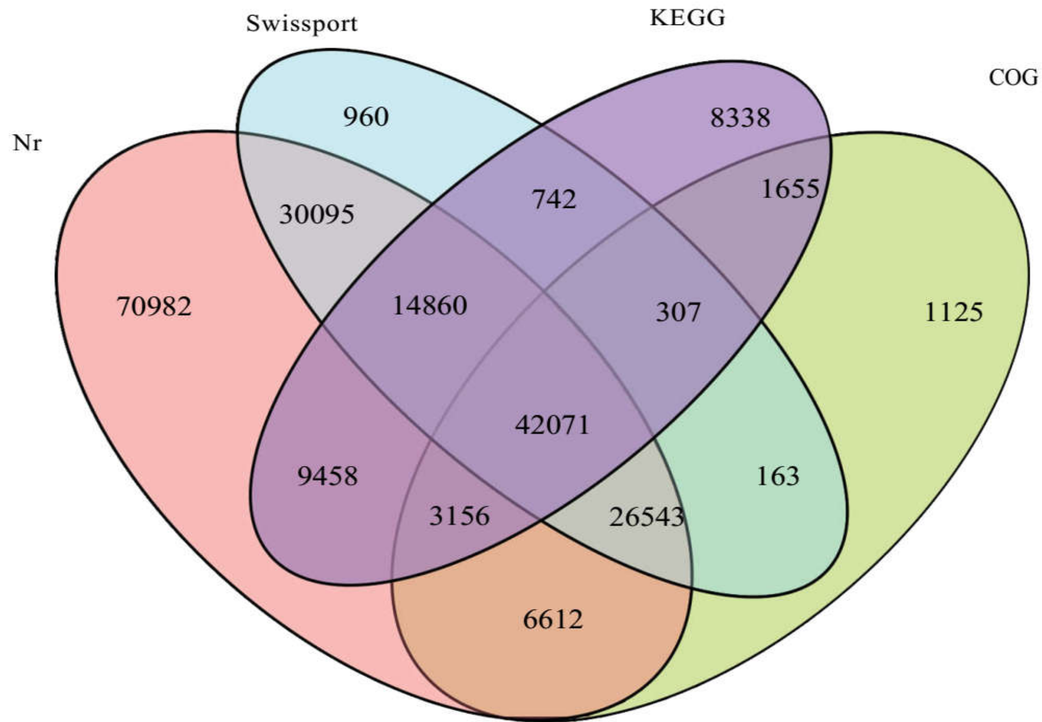

Figure 3: Venn diagram showing annotation of unigenes

3.2.2 GO and COG annotation analysis

The most over-represented GO terms in response to drought and salt stresses were “binding, 48,000 unigenes”, “catalytic activity, 39,000 unigenes” both under molecular function category. “Cell part, 30,000 unigene”, “organelle 28,000 unigenes” from Cellular component and “Metabolic processes 30,000 unigene”, “cellular process 28,000 unigenes” from the biological process were also represented with a significant amount of unigenes (**Figure 4**).

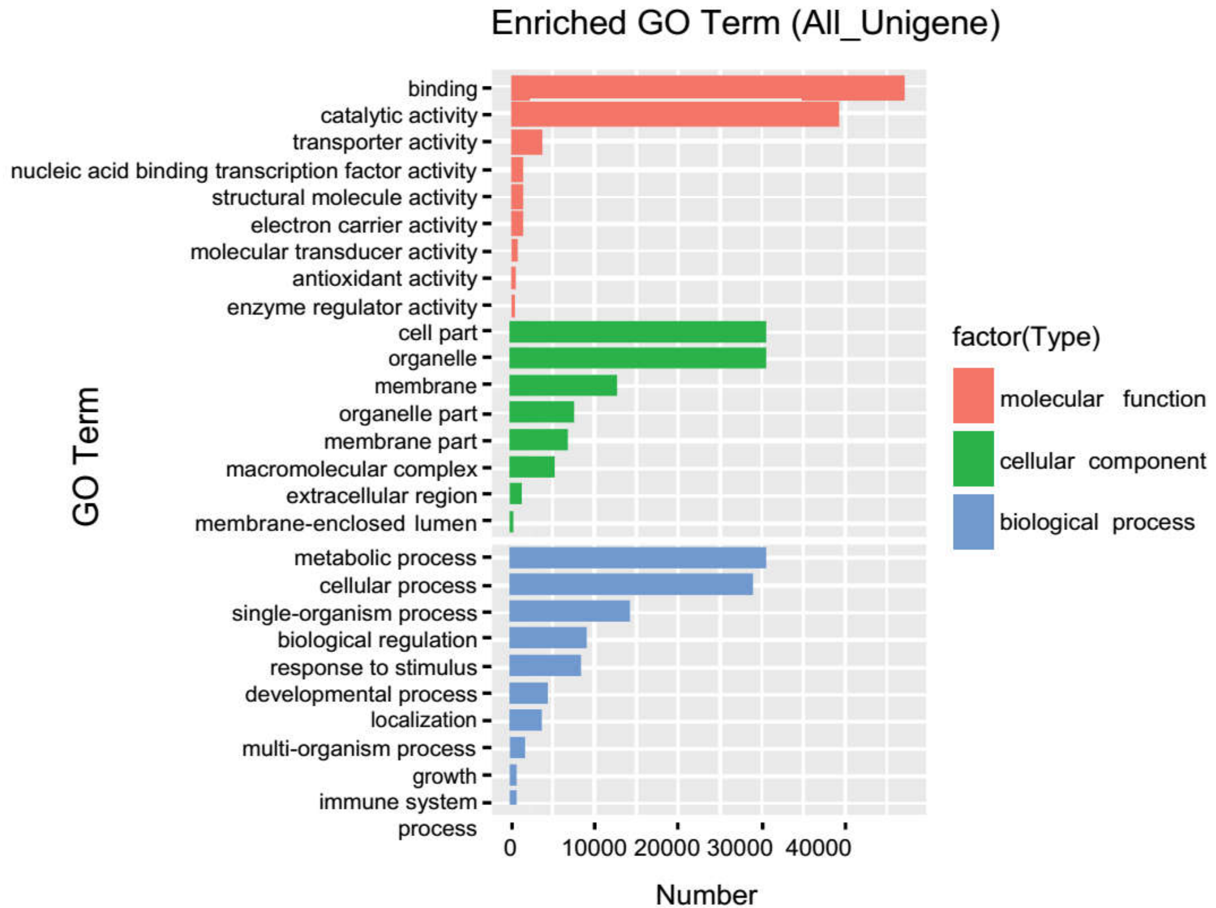

Figure 4. Gene ontology classification of lowland bamboo unigenes

The vertical axis is the enriched GO terms and the horizontal axis is the number of unigenes in each term. Different colors were used to distinguish between biological processes, cellular components and molecular functions.

Clusters of Orthologous Groups (COG) database annotation searches of 406,181 unigenes have predicted 81, 632 unigenes (20%) that were assigned into 25 functional categories. The most dominantly represented functional subcategories were “Signal transduction mechanisms” with 12,861 (15.7%) unigenes followed by “Posttranslational modification, protein turnover, and chaperones” with 10,191 unigenes (12.5%) which belongs to cellular processing and signaling

pathway categories which makes 28 % of the total functional annotations. The third most represented was "General function prediction only" with 9993 unigenes (12.2%) followed by "Intracellular trafficking, secretion and vesicular transport" with 4968 unigenes (6%). Among the unigenes categorized into specific COG functional annotations, 4,933 unigenes (6%) were categorized under "function unknown" (**Table 2**).

Table 2: COG functional classifications of lowland bamboo transcripts.

Code	Description	Gene num
	Metabolism	
C	Energy production and conversion	3803
Q	Secondary metabolites biosynthesis, transport and catabolism	2833
P	Inorganic ion transport and metabolism	2480
F	Nucleotide transport and metabolism	1072
I	Lipid transport and metabolism	3430
H	Coenzyme transport and metabolism	980
G	Carbohydrate transport and metabolism	4848
E	Amino acid transport and metabolism	4136
	Information storage and processing	
L	Replication, recombination and repair	2240
B	Chromatin structure and dynamics	1179
A	RNA processing and modification	3702
K	Transcription	4928
J	Translation, ribosomal structure and biogenesis	4987
	Cellular processing and signaling	
O	Posttranslational modification, protein turnover, chaperones	10191
W	Extracellular structures	266
Z	Cytoskeleton	2343
M	Cell wall/membrane/envelope biogenesis	1328
N	Cell motility	38
D	Cell cycle control, cell division, chromosome partitioning	2066
T	Signal transduction mechanisms	12861
Y	Nuclear structure	250
V	Defense mechanisms	722
U	Intracellular trafficking, secretion, and vesicular transport	4968
	Poorly characterized	
S	Function unknown	4933

3.2.3 Metabolic pathway analysis

To understand the biological functions of the unigenes on the biochemical pathways, KEGG functional classification was performed and obtained 80,587 (19.8%) unigenes involved into 32 KEGG pathway categories. Among the KEGG pathways categories, signal transduction is the most represented with 25,000 genes (31%) followed by carbohydrate metabolism 12,000 genes (14.9%). The other pathway categories with a significant number of unigenes include Endocrine system, global and overview maps, translation, folding, sorting, and degradation pathway categories. These pathways have unigenes ranging from 8,000 to 9,000 genes (**Figure 5**).

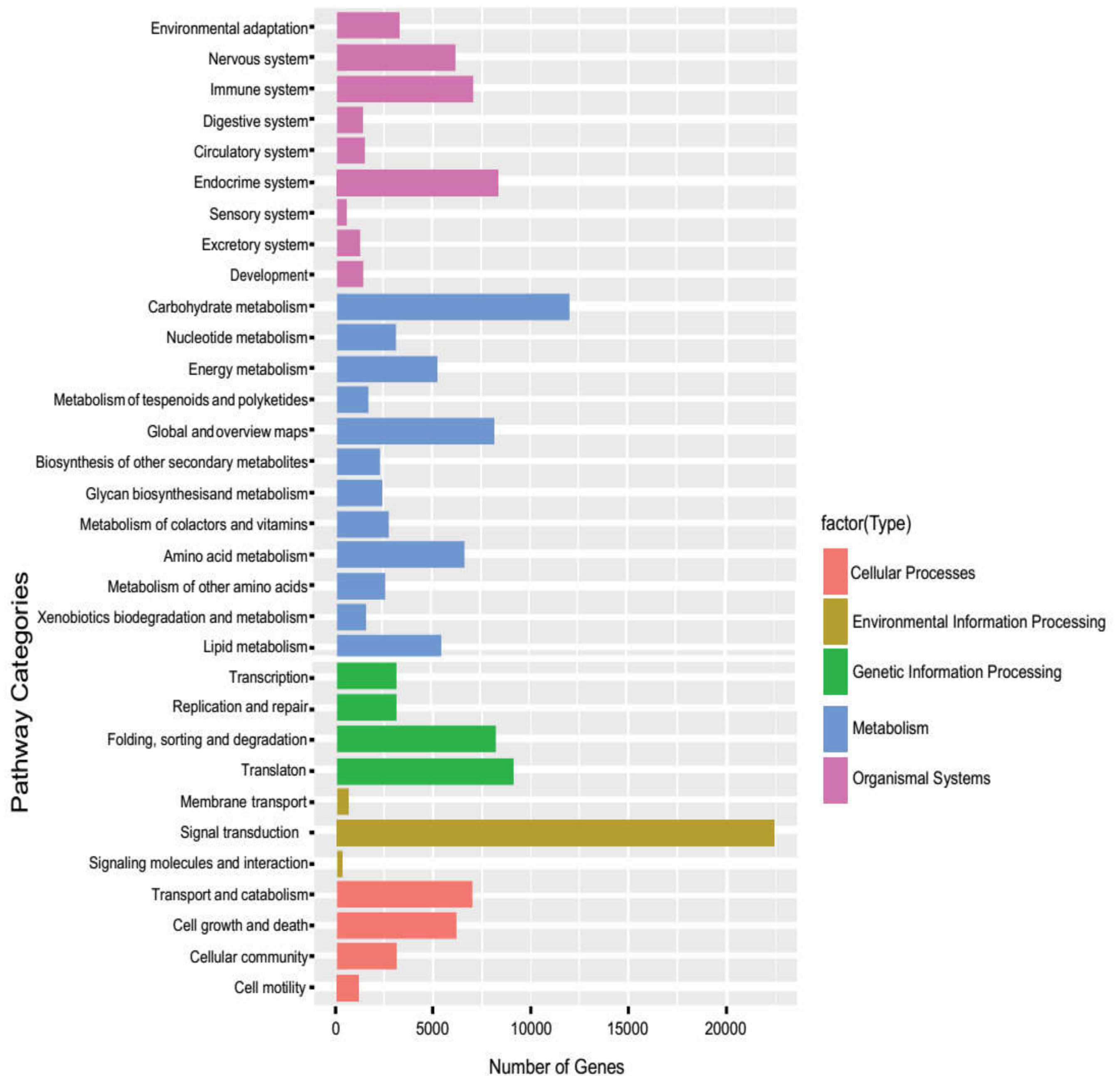

Figure 5: KEGG classifications of lowland bamboo unigenes

The vertical axis for biological pathways for the five categories; horizontal line for the number of genes; different colors used to distinguish the biological pathway of a classification.

3.2. 4 Prediction of transcription factors and protein family assignment

Transcript factors have been exhibited to engage in key tasks in reaction to abiotic stresses by regulating gene expression (Nuruzzaman *et al*., 2013; Nakashima *et al*., 2014). For a comprehensive understanding of lowland bamboo's gene control and regulation, transcript factors were predicted using family assignment rules established at http://itak.feilab.net/cgi-bin/itak/online_itak.cgi (Zheng *et al*., 2016). According to family assignment rules 4,332 transcript factors (TFs) gene families were predicted to have active involvement in the regulations of gene expression and these TFs were organized into 64 transcription factors. WD40, NAM/NAC, WRKY, bHLH, AP2/ERF, MYB relate and bZIP, were the most dominant TFs families (**Figure 6**). In this study the most represented TF families with differentially expressed and protein coding genes (DEGs) includes, bZIP (49), WRKY (43), MYB (38), AP2/ERF (30), HD-ZIP (25) and MYB related (21), which demonstrates members of these gene families are closely associated with abiotic stress defense (Deinlein *et al*., 2014; Zhao *et al*., 2016).

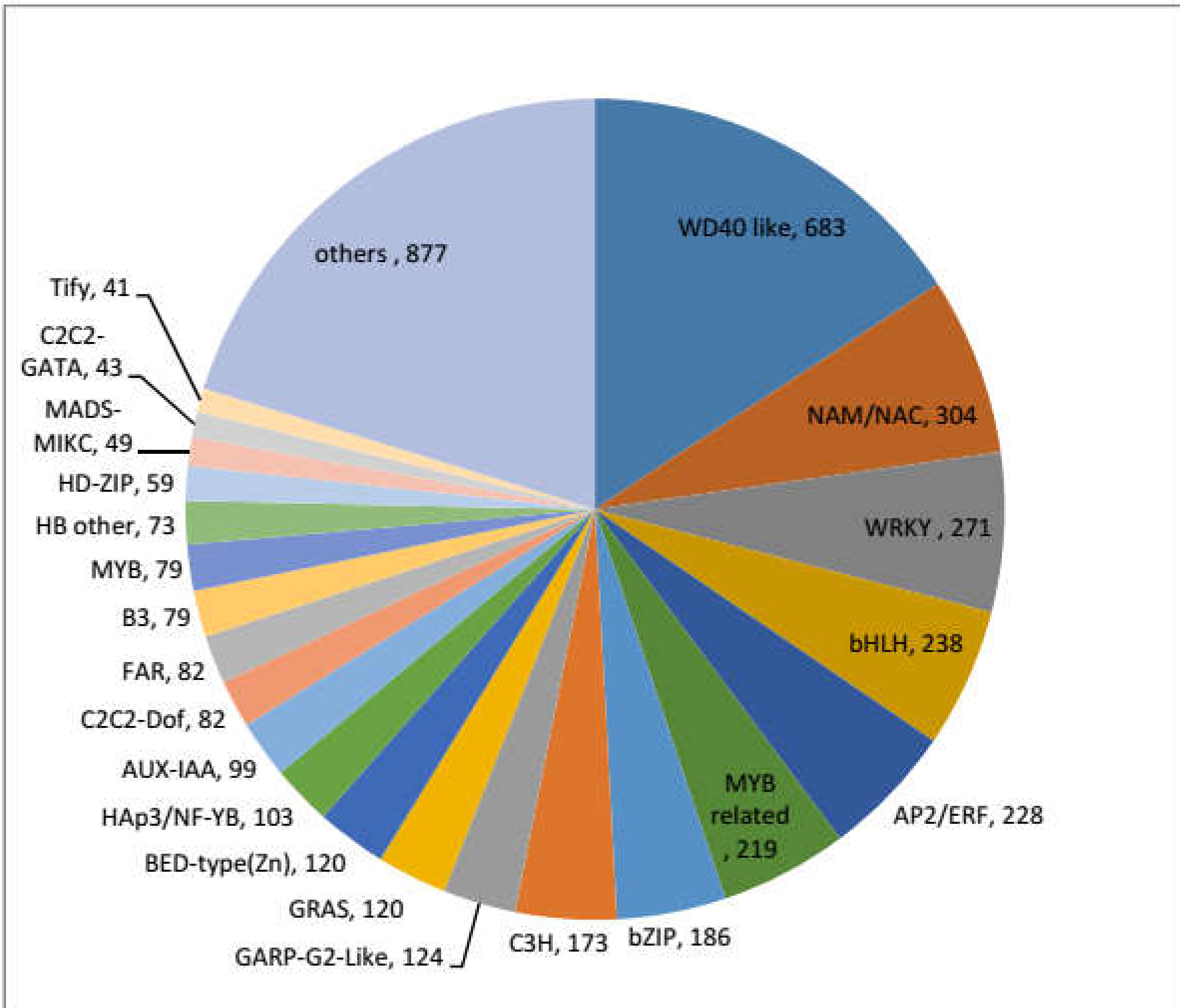

Figure 6: The most abundant predicted transcription factor families

3.2. 5 Gene expression and differential expression genes (DEGs) analysis

The direct expression of a gene's expression level is the abundance of its transcript, the higher the degree of transcript abundance, the higher the gene expression level. DEGs analysis used three combinations: (i) Control vs. drought, (ii) control vs. salt and (iii) drought vs. salt. The numbers of DEGs common and specific between the different stresses were presented at **Figure**

7. Clustering analysis was conducted to: (i) calculate the similarity of the data and classify the data according to the similarity so as to cluster together the genes with the same function or close relationship, (ii) to identify the unknown gene function or the unknown function of the known gene and (iii) to infer whether they commonly participate in the same metabolic process or cell pathways.

Differential expression genes (DEGs) analysis from this abiotic stress-induced transcriptome study resulted into the expression 65,471 stress-responsive genes, 29,746 up-regulated and 35,725 down-regulated. This indicated that these DEGs are involved in regulating the response to drought and salt stresses. Due to drought stress, a total of 34,719 DEGs were identified, among these 15,681 were up-regulated and 19,038 were down-regulated. Out of 13,958 salt24h stress DEGs, 5,994 up-regulated and 7, 964 down-regulated. Out of 3,878 salt48h DEGs, 1, 289 were up-regulated and 2,589 were down-regulated. Out of 12,916 droughts vs. salt DEGs, 6,782 were up-regulated and 6,134 were down-regulated.

In both drought and salt stresses when compared to control, the numbers of down-regulated DEGs are greater than up-regulated ones. But in the case of drought vs. salt DEGs analysis, up-regulated genes are greater. When we observe DEGs among treatments, genes co-expressed exclusively in each stress includes 15,745 for control vs. drought, 2,118 for control vs. salt 24 h, 910 for control vs. salt 48 h and 1,875 for salt vs. drought. But 25,150 DEGs were shared by two or more groups of treatments while 569 were shared by all stress groups (**Figure 7**).

Out of 65,471 DEGs, 14, 258 (21.8%) are functionally annotated. Most importantly 78.2 % of the DEGs were not classified to any functional pathways, which suggests these putative novel genes might be species-specific and these genes might be involved in key pathways regulating in stress adaptation.

Out of 29, 746 up-regulated genes, only 7,103 (23.8%) could be assigned to putative functions. Some genes have a wide range of functions by involving in dozens of pathways. For instance, in drought stress, GO: 0000165, GO: 0005524 GO: 0080136, each involved in 97 pathways. In salt 24h stress GO: 0080136 (97), GO: 0010099 (37), GO: 2000021 (23). In salt 48 h, GO: 0005739 involved in 18 pathways. In drought vs. salt, GO: 0080136 in 97 pathways.

Out of 35,725 down-regulated genes, only 7,155 (20%) were assigned to functions. The down-regulated genes with the most diverse functions by participating in dozens of pathways includes, in drought stress, GO: 0000165, GO: 0005634, GO: 0042542 and GO: 0005524 all under the term mitogen-activated protein kinase involves in 97 pathways, GO: 0005952 in 63 pathways. In salt24, GO: 0005952 (64), GO: 0005509 and GO: 0009536 each in 37 are with the most diverse functions. In salt48h, GO: 0004721 (21) are the most multi-functional genes. In drought vs. salt, GO: 0007264 (41).

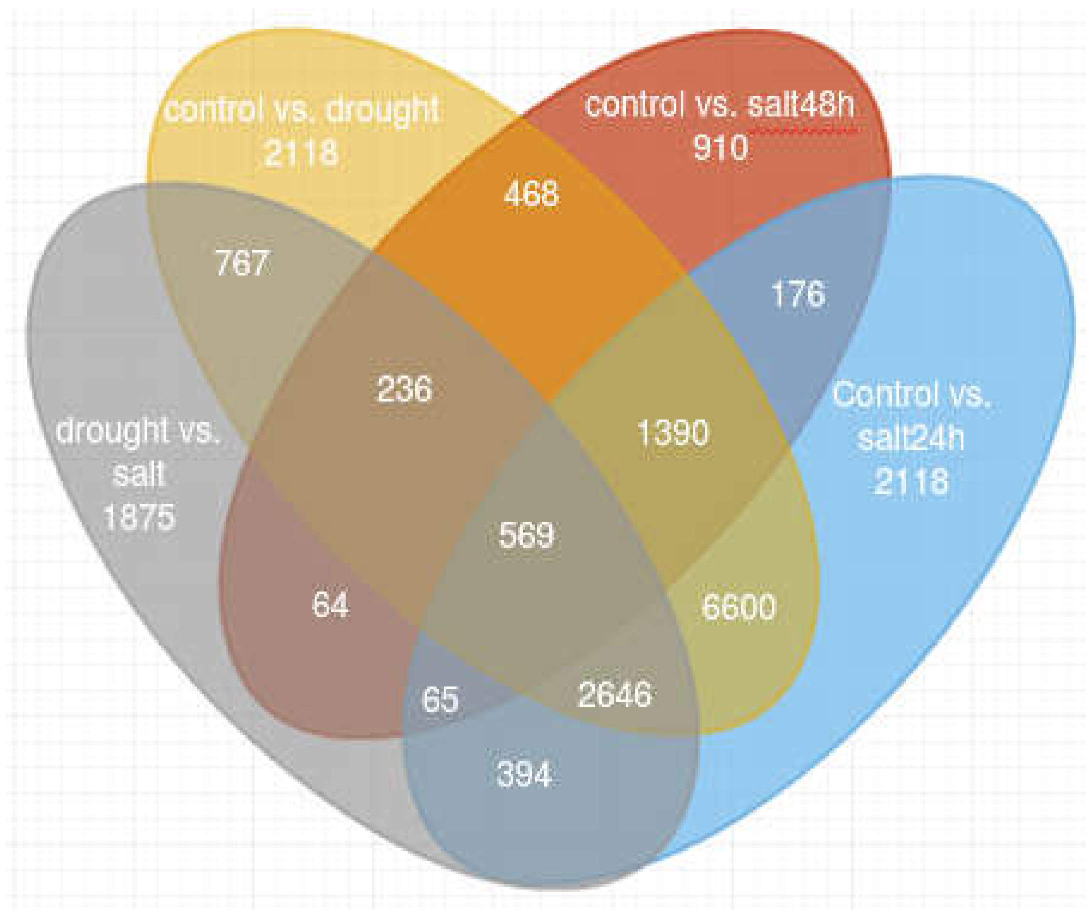

Figure 7. Venn diagrams showing the number of differentially expressed genes in each pair of samples, as well as the number of shared differentially expressed genes.

Treatment groups stands for N1-3 vs. P1-3(drought vs. salt), W1-3 vs. P1-3(control vs. drought), W4-6 vs. N4-6 (control vs. salt48h) and W1-3 vs. N1-3(Control vs. salt24h).

3.2.6 Verification of RNA-Seq data by qRT-PCR

In order to confirm the reliability of the RNA-Seq data, RT-qPCR was performed. Randomly selected 20 ups- regulated and 20 down-regulated genes were used. To validate the expression levels measured by RNA-Seq, the ratio of expression of selected genes as measured by qRT-

PCR was compared to the ratio of expression under drought and salt stress conditions using RNA-Seq. From 40 selected unigenes, only 13 unigenes could be quantified due to specificities of primers. But because of sample replications these unigenes were quantified from 28 gene samples. The RNA-Seq data have strong positive correlation coefficient value with qRT-PCR, since the value of $R^2 = 0.90$ (Appendix **4**).

3.2.7 Data deposition

The raw sequencing reads are deposited in the NCBI Sequence Read Archive (SRA) under SRP153816 accession number and the assembled transcripts are also deposited in the Transcriptome Shotgun Assembly (TSA) Database, under GGTK00000000 accession number.

3.2.8 Underlying mechanisms associated with abiotic stress tolerances of lowland bamboo

RNA-Seq global transcriptome analysis of lowland bamboo provided important insights into the gene regulatory mechanisms used by the plant in the face of drought and salt stress. Such global expression analysis helps to uncover the adaptive genes, pathways and regulatory mechanisms that are important for drought and salt tolerance traits.

Identifying the most represented pathways by assigning genes to their functional categories is very important to unveil the underlying molecular mechanisms behind lowland bamboo abiotic stress tolerance in general and drought and salt stress in particular. According to metabolic pathway analysis, the most important pathways with their underlying mechanisms in enhancing plant tolerances to abiotic stress are presented in **Table 3**.

Table 3 .The most important pathways with their underlying mechanisms for abiotic stress tolerance.

GO terms	Pathways	Underlying mechanisms to improve abiotic stress tolerances
Up-regulated drought		
GO:0000165	MAPK cascade	transmit a signal within a cell
GO:0031410	cytoplasm to vacuole targeting (Cvt) pathway	Sequestration by vacuoles
GO:0007264	intracellular signaling pathway	intracellular signal transduction
GO:0005509	cell signaling pathway	interacting selectively and non-covalently with any ion
GO:0004674	Protein kinase pathway	catalysis of reactions
GO:0000166	TA-proteins (GET) pathway	binding small molecules
GO:2000021	SOS pathway	maintenance of ions
GO:0005513	signal transduction pathways	detection of chemical stimulus
Down-regulated drought		
GO:0009814	Calcium dependent protein kinase (CDPK) pathway	prevents the occurrence or spread of disease
GO:0005886	endocytic pathway	cell surrounding and protection
GO:0004721	complex phosphorylation signaling pathways	catalysis of reactions
Up-regulated salt		
GO:0009651	Salt Overlay Sensitive (SOS) pathway	Exclusion of Sodium ion from the plant root

3.3. Discussion

To date, there is no data pertaining to transcriptome properties of lowland bamboo under abiotic stress. The lack of reference genomic data has significantly hampered research on the species. Transcriptome sequencing using Illumina Seq platform enabled to create relatively comprehensive sequences data that can be utilized for research including comparative study, gene cloning, expression analysis and EST-SSR markers development.

The average length of 641 bp, N50 of 873 bp was more or less comparable with other *de novo* assembled transcriptome in other plant species (Liu *et al*., 2012; Wu *et al*., 2014; Li *et al*., 2016; Muller *et al*., 2017; Salgado *et al*., 2017). On the contrary *de novo* assembled hexaploid hulless oat has generated 128,414 putative unigenes (Wu *et al*. 2017). Such deviation might be due to treatment as oat was treated with salt only. Transcriptome analysis of five hexaploid and allododecaploid spartina species generated unigenes ranging from 13, 054 to 16,002 (Boutte *et al*., 2016). In the species similarity search, lowland bamboo showed less similarity with Moso bamboo. This genetic divergence might be due to the existences of more than 1, 250 species of bamboos across the globe, differences in speciation time, variation in selection pressure and geographic distances.

When compared to transcriptome analysis of other plants, the 217,067 number of functionally annotated unigenes of lowland bamboo were much higher. Although the number is higher, the percentage of annotated unigenes is smaller (53.4%). For instance, the number of unigenes and annotated percentage of some species includes, 59, 814 unigenes (73%) in drought and cold

stresses response of *Ammopiptanthus mongolicus*, 177, 817 unigenes (94.8%) in salt response of *Spartina alterniflora*, 54,125 unigenes (58.7%) in desiccation-tolerant moss *Syntrichia caninervis*, 95,897 unigenes (83.57%) in *Miscanthus sinensis* (Liu *et al*., 2012; Wu *et al*., 2014; Gao *et al*., 2014; Bedre *et al*., 2016).

The GO and COG functional classification analysis of all unigenes provide valuable information in predicting possible gene functions and in determining the gene function distribution of unigenes. In lowland bamboo, of the total 406, 181 unigenes, 217,067 (53. 4 %) unigenes were functionally annotated. The remaining 189,114 (46.6 %) unigenes did not show any significant functional similarity to any of the database explored. This may be attributed to the lack of references genome of lowland bamboo and the highly divergent nature of the unigenes or novel genes that carry out species-specific functions. The GO analysis revealed that binding and catalytic activity from molecular function and metabolic processes from the cellular component were represented with a high number of unigenes. More or less similar supporting results were found in transcriptome analysis of *Ammopiptanthus mongolicus*, *Nitraria sibirica* and *Urochloa decumbens* under abiotic stresses (Wu *et al*., 2014 ; Li *et al*., 2016 ; Salgado *et al*., 2017), which suggests that genes involved in the above GO terms are responsive to abiotic stress.

Only 20 % of unigenes were assigned to COG database and the majority of these unigenes goes to cellular processing and signaling pathway categories. These two dominant subcategories were also with the highest number of unigenes in a transcriptome analysis of Moss, Tall Fescue and Tobacco (Gao *et al*., 2014; Talukder *et al*., 2015; Li *et al*., 2016). This suggests large numbers

of genes in cellular processing and signaling are actively involved in drought and salt stresses response.

Functionally unclassified 4,933 unigenes might stand for lineage and/or species-specific genes for adaptive innovation and could be un-translated regions of the transcriptome or novel genes that perform species-specific functions. Signal transduction mechanism at COG and signal transduction at KEGG were represented by the highest numbers of genes, which suggests that both the genes involved and the functional pathways actively respond to stress (Wu *et al*., 2014). The metabolic pathways analysis result suggests that signal transduction and carbohydrate metabolism events are active in lowland bamboo response in the face of drought and salt stress. Similar supporting results were obtained from Cotton and Buckwheat transcriptome analysis under abiotic stress (Wu *et al*., 2017; Zhu *et al*., 2013).

Transcript factors or sequence specific DNA-binding proteins are believed to play decisive role in stress signal transduction pathways. TFs families with proven roles in regulation of stress response in plants include, NAC, WRKY, AP2/ERF, bZIP, and MYB related (Naika *et al*., 2013). TFs showing up-regulation for both stresses could be targets for studying plant response in the face of unfavorable environmental conditions. Whole genome expression profiling and transcriptome studies in many plants have confirmed the stress responsiveness of various TF families, C2H2 in *Populus trichocarpa* (Liu *et al*., 2015), NAC in soybean (Le *et al*., 2011), WD40 in foxtail millet (Mishra *et al*., 2014), WRKY in carrot (Li *et al*., 2016) and broomcorn millet (Yue *et al*., 2016). In addition to wild plants, many studies have been conducted on the role of TF families in stress response of transgenic plants, like AP2/ERF conferred stress

tolerances in *Populus simonii* x *Populous nigra* (Yao *et al*., 2017), manipulation of specific NAC TFs has conferred stress tolerance in transgenic rice, tobacco, wheat and *Arabidopsis* (Shao *et al*., 2015), AP2/ERF in transgenic *Trifolium alexandrinum* (Abogadallah *et al*., 2011), bHLH in *Arabidopsis thailina* (Babitha *et al*., 2013). The involvements of TF families in diverse abiotic stresses have been reported in different plants (Wani *et al*., 2013; Joshi *et al*., 2016; Wang *et al*., 2016).

Analyses of differentially expressed genes have revealed abiotic stress responsiveness of lowland bamboo genome. Although drought stress has one technical replication, the numbers of its DEGs are almost double compared to salt stresses which have two technical replications. This suggests that lowland bamboo might be more responsive to drought than salt stress. As 569 DEGs were shared by all stresses groups, this genes might be blessed in conferring multiple stress tolerance so that those genes might serve as potential candidates for exploring multiple stress tolerance and adaptation (Ziaf *et al*., 2011; Thapa *et al*., 2016 ; Jawaher, 2018). A total of 65,471 DEGs, from these 29,746 up-regulated and 35, 725 were down-regulated. DEGs analysis of hexaploid hulless oat has generated 65,000 unigenes (Wu *et al*., 2017). The functionally annotated 14, 258 genes proved to be actively involved in diverse pathways activities. As many genes involved in similar functions, the most represented proteins by up-regulated genes include, heat shock 70kDa protein 1/8, serine/threonine-protein kinase SRK2, protein phosphatase 2C, beta-glucosidase, glutathione S-transferase protein xylosyltransferase, GTP-binding nuclear protein Ran, CTP synthase, adenylate kinase, Delta 1-pyrroline-5-carboxylate Synthetase, glutamine synthetase, Chitinase and glyceraldehyde-3-phosphate dehydrogenase. Majority of the above proteins take part in an important role in the regulation of stress tolerance. Serine-

threonine protein kinases are proven to take part in the regulation of signaling cascades and some of these when over-expressed improved stress-tolerance of plants (Mao *et al*., 2010). Glutamine synthetase involves in incorporating toxic free ammonium ions into glutamate and glutamine, respectively; thus up-regulation of these genes may be a possible mechanism of stress tolerance (Skopelitis *et al*., 2006). Largely represented up-regulated genes like Delta 1-pyrroline-5-carboxylate Synthetase, glyceraldehyde-3-phosphate dehydrogenase and Chitinase play important role in plant defense and enhance resistance and tolerance to drought and salt stresses (Wu *et al*., 2014; Magrane *et al*., 2015).

The most prevalent proteins from lowland bamboo transcriptome analysis encoded from down-regulated DEGs includes, interleukin-1 receptor-associated kinase 4, aldehyde dehydrogenase (NAD+), fructose-bisphosphate aldolase, phosphoenolpyruvate carboxylase, cysteine synthase A, (S)-2-hydroxy-acid oxidase, glutamate synthase (ferredoxin), thioredoxin reductase (NADPH) and light-harvesting complex II chlorophyll a/b binding protein. Among the down-regulated genes, with a significant role in stress tolerances were GABA transporter, arginine, which is one of the precursors of putrescine, glutamate which is the precursor molecule for proline (an osmo-protectant) plays important role in plant stress-regulation and stress- tolerance (Shi and Chan, 2015). Phosphatase 2C family protein also played a significant role in stress tolerance (Lee *et al*., 2013). Receptor protein kinase-like and Glucose-6-phosphate dehydrogenase are also believed to confer stress tolerances in barley dehydration shock and drought stress (Talame *et al*., 2007).

One of the most important aspects of global transcriptome profiling is to link genes to their possible molecular mechanisms in assisting plants against abiotic stress. Identifying the most represented pathways by assigning genes to their functional categories is very important to unveil the underlying molecular mechanisms behind plants' drought and salt stress tolerances in general and lowland bamboo abiotic stress tolerance in particular. Interestingly majority of pathways unveiling the underlying mechanisms that contribute to improved abiotic tolerances were found at both drought and salt treatment groups. This suggests that genes involved in such pathways could be used to engineer plants for both traits. Some of the key mechanisms like detoxification, sequestration by vacuoles and cell wall modification were also revealed in stress response of rice against salt (Wang *et al*., 2015).

CHAPTER 4

4. Genome-wide analysis and expression profile of bZIPs gene family in lowland bamboo under drought and salt stress.

4.1 Materials ad Methods

4.1.1 Gene ontology and functional classification

The materials and methods is presented at Chapter three of this paper. To obtain the GO annotation against Eukaryote Protein sequences of bZIP genes were loaded into the CELLO2GO http://cello.life.nctu.edu.tw/cello2go/ (Yu *et al*., 2014). Genes were also categorized as per the GO biological process, its molecular function and cellular component according to CELLO2GO GO functional classification. Metabolic pathway analysis was conducted using BlastKOALA tool (https://www.kegg.jp/blastkoala/) (Kanehisa *et al*., 2015).

4. 1.2 Phylogenetic analysis

Phylogenetic analysis was performed using 162 bZIP proteins. ClustalX program was used to perform multiple sequences alignment of protein sequences. Maximum parsimony method was used to construct an un-rooted phylogenetic tree by using MEGA X(Kumar *et al*., 2018).The Maximum parsimony tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm.

4.2 Results

4.2.1 Identification of bZIP TFs Gene Family in lowland bamboo genome

Previously, we established drought and salt stress induced global transcriptome analysis of lowland bamboo (Adem *et al*., 2019). As a result of the experiment 406,201, *de novo* assembled unigenes of lowland bamboo were obtained and served as a source for identification of bZIP

unigenes used in this particular study. To identify bZIP genes, deduced protein sequences of lowland bamboo were submitted to the plant transcription factor database, http://itak.feilab.net/cgi-bin/itak/online_itak.cgi (Zheng *et al*., 2016). The search resulted into the identification of 186 putative bZIP proteins. To confirm the presences of the conserved bZIP DNA binding domain (PF00170), the resulting protein sequences from iTAK database were further subjected to Pfam analysis (https://pfam.xfam.org/), then 162 proteins were confirmed to be under the bZIP binding domain.

The lowland bamboo bZIP (*OabZIP*) genes encoded proteins that vary in their sizes and sequences. The length of Proteins of *OabZIPs* ranges from 77 to 1840 amino acids. The molecular weights of OabZIPs were conducted using the ExPASy database (http://expasy.org/). Accordingly, the *OabZIPs* protein sequences had higher variations in molecular weight which ranges from Glycine having 75.07 kilo Dalton to Tryptophan 204.23 kilo Dalton.

4.2.2 Gene ontology and Pathway analysis

According to CELLO sub-cellular localization predictor analysis, all the bZIP unigenes were localized to the five cellular parts. Majority of the bZIP unigenes are found in nuclear compartment, 114 of the unigenes, followed by 36 localized to the plasmamembrane. The remaining sub cellular localization shares a small number of bZIPs as shown in **Figure 8**.

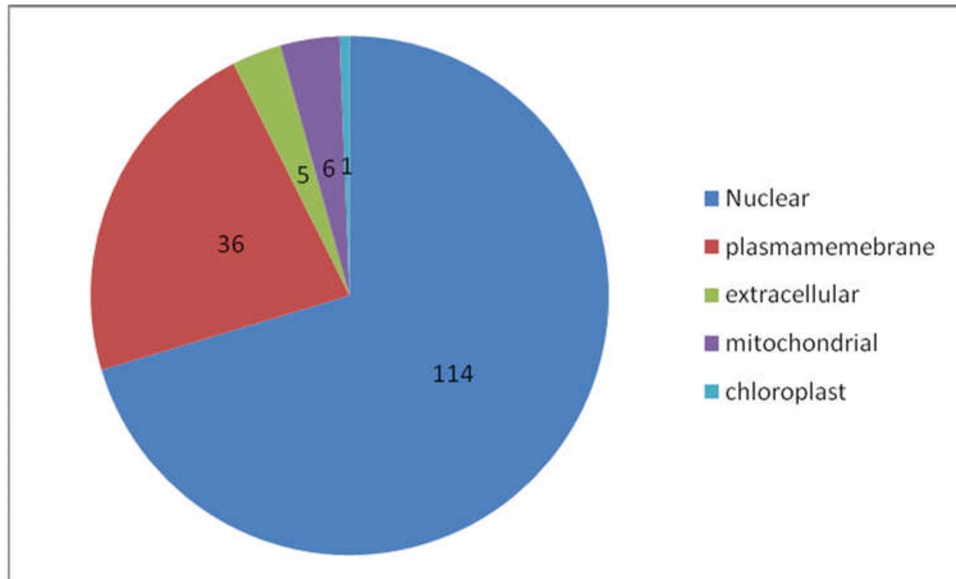

Figure 8: Sub-cellular localization of 162 unigenes of bZIP transcription factor family.

Gene ontology enables to identify those genes involved in the biological process, those enabling molecular function and those who are part of the cellular component. Using CELLO2GO gene ontology analysis, cellular component covered 140 (86.41%) of unigenes, which tells that 22 of the bZIP unigenes are out of the cellular component. The molecular function has covered 141 (87.03%) unigenes out of 162 unigenes, which informs that almost 21 of the bZIPs have no role in molecular function. Biological process analysis has covered 137 (84.56%), which unveils that the remaining 15.44 % of unigenes did not show any significant biological functional similarity to the explored database.

As it's depicted in **Figure 9**, organelle, intracellular, cell, and nucleus were represented by an almost equal number of unigenes. Those ontologies with a significant role in enabling molecular function were DNA binding and nucleic acid binding transcription factor activity. When compared to cellular component (nine ontologies) and molecular function (six ontologies), a number of ontologies with diverse functions were involved in the biological process (16

ontologies). The most represented ontologies in the biological process were cellular nitrogen, biosynthetic process, and signal transductions most importantly about 16 unigenes were involved in response to stress.

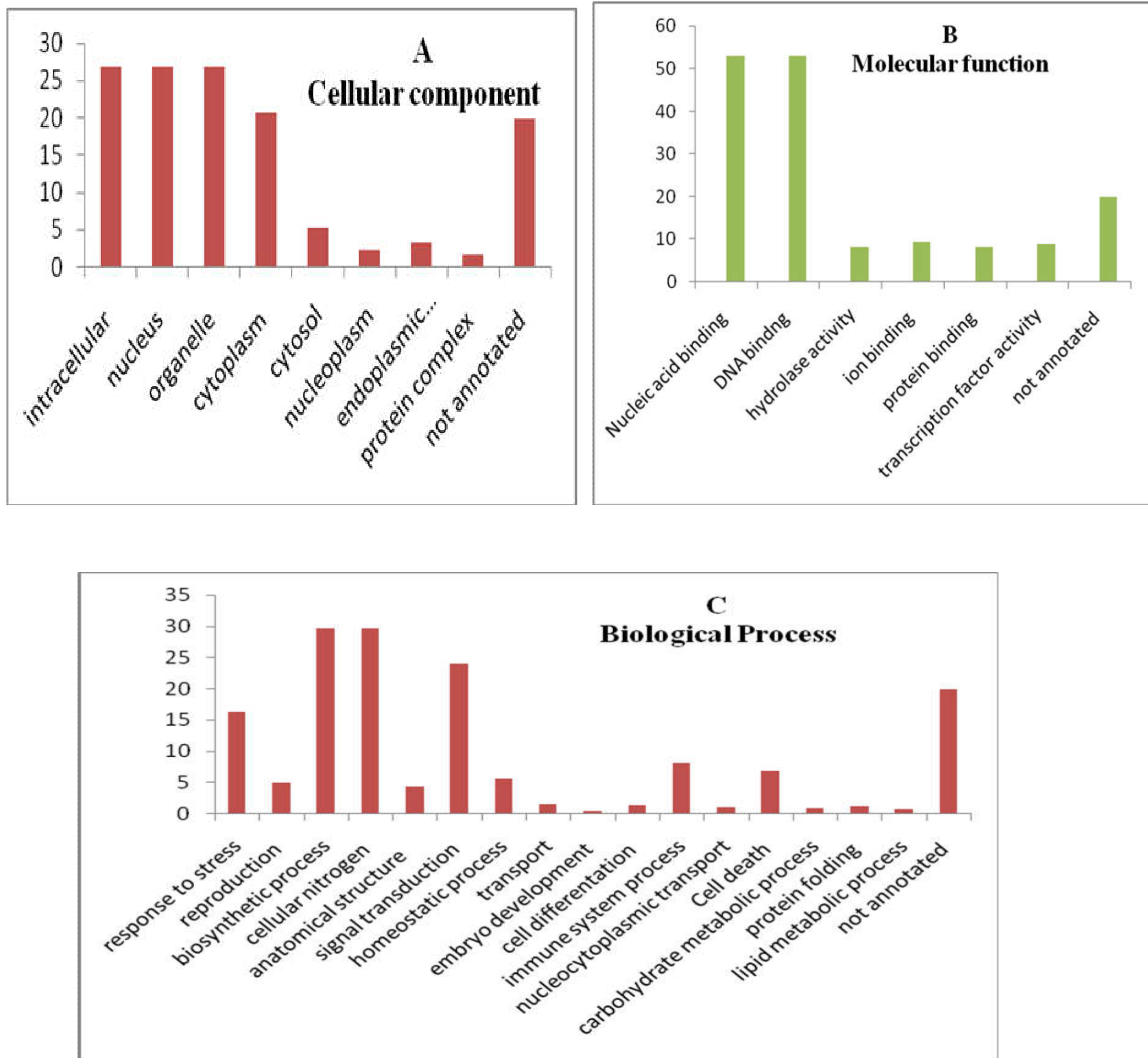

Figure 9: Gene ontology classification of bZIP transcript factors. A. Cellular Component, B. Molecular function and C. Biological process.

As it's shown in **figure 10**, among the162 unigenes of bZIPs, only 34 (21%) were annotated. The metabolic pathway analysis revealed that environmental information processing and genetic

information processing categories were the only represented pathways. This uncovers that bZIPs transcript factor families are actively involved in stress regulation. Although bZIPs transcript factors activity is limited to the two pathway categories, these two categories are highly associated with stress response and regulation which implies that bZIPs might be highly associated with stress response.

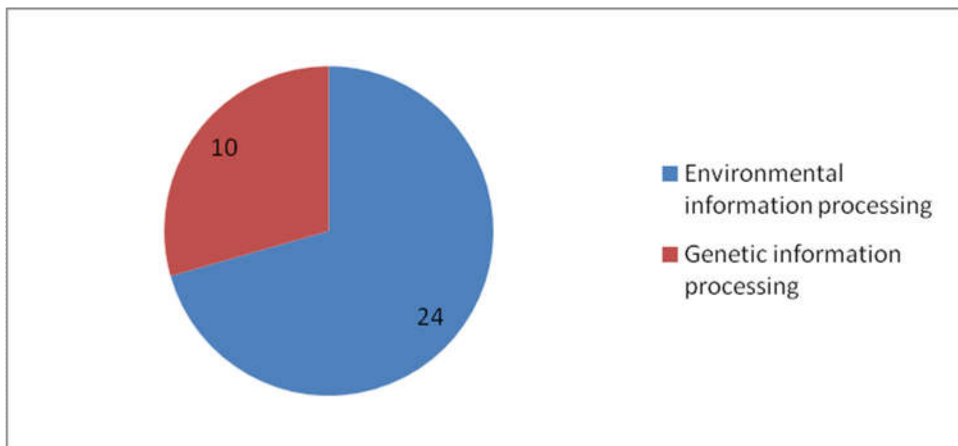

Figure 10: Pathway analysis using the Blast KOALA tool.

4.2.3 Expression analysis of bZIP genes under drought and salt stresses

Among the identified 162 expressed genes of bZIPs, 149 were differentially expressed genes (DEGs), 99 up-regulated while 50 were down regulated and 13 genes were not differentially expressed i.e. their level of expression is affected by neither by drought nor by salt. From such differentially expressed genes, 49 genes were functionally annotated while the functions of the remaining genes were not unveiled. The most represented functionally annotations of bZPs in response to drought and salt stress in lowland bamboo were salt-stress inducible bZIP proteins and abscisic acid-insensitive (ABA) as shown in **Table 4**.

Table 4: DEGs and functional analysis of selected up-regulated bZIP transcript factors

Gene ID	|log2FoldChange|	GO_ID	KO_Definition
TRINITY_DN145821_c6_g2_i2	3.070293243	-	transcription factor TGA
TRINITY_DN150849_c2_g1_i9	2.332884004	GO:0003677	plant G-box-binding factor
TRINITY_DN140653_c0_g1_i1	2.9684483	-	ABA responsive
TRINITY_DN145058_c5_g2_i4	8.1826365	GO:0006355	ABA responsive element binding -factor
TRINITY_DN152701_c1_g1_i1	7.4411664	GO:0003700	ABA responsive element binding factor
TRINITY_DN153711_c3_g9_i3	7.4033188	GO:0003700	ABA responsive element binding factor
TRINITY_DN150849_c2_g1_i10	6.7880783	GO:0003677	plant G-box-binding factor
TRINITY_DN145821_c6_g2_i4	1.9251616	-	transcription factor TGA
TRINITY_DN145821_c6_g2_i3	3.8560311	GO:0005634	transcription factor TGA
TRINITY_DN143358_c12_g3_i3	3.26747	GO:0005634	transcription factor HY5
TRINITY_DN153556_c2_g1_i6	1.2501836	GO:0006355	plant G-box-binding factor
TRINITY_DN143358_c12_g3_i5	2.0792177	GO:0005634	transcription factor HY5
TRINITY_DN126016_c3_g2_i1	3.15973622	GO:0006355	salt-stress inducible bZIP protein

4.2 .4 Phylogenetic analysis

The phylogenetic analysis was conducted to understand the evolutionary implication of bZIPs in lowland bamboo. The phylogenetic tree was constructed using 162 *OabZIP* proteins by employing maximum parsimony method and bootstrap values from 1000 replicates. The phylogenetic analysis categorized bZIP proteins into14 distinct groups from cluster I to XIV. Number of proteins in each cluster significantly varies, cluster III and cluster VII each with one Protein and cluster XIV with 28 proteins (**Figure 11**).

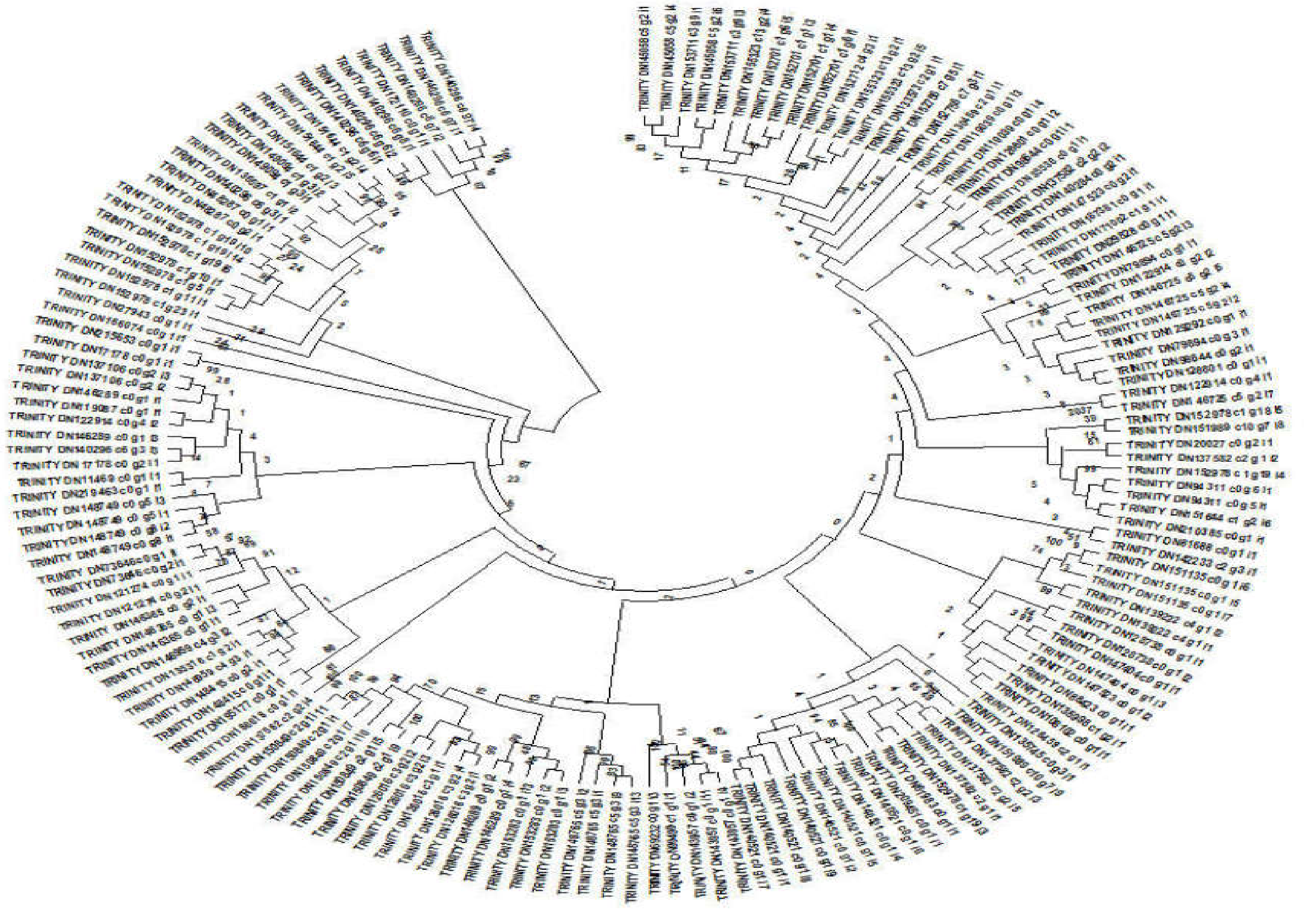

Figure 11: Phylogenetic tree of 162 aligned bZIP subfamily proteins in lowland bamboo.

4.3 Discussion

Transcription factors are proteins regulating gene expression that have received special attention from scholars engaged in plant whole-genomic sequencing and transcriptome sequencing. Drought and salinity are among the key factors that cause plant failure. Among the different transcription factors, previous studies have shown bZIP transcription factors were highly associated to plant stress response (Wang *et al*., 2011; Wang *et al*., 2017).

This study has identified 162 bZIPs, compared to that of 64 in *Syntrichia caninervis* (Gao *et al*., 2014), 17 in *Chlamydomonas reinhardtii* (Ji *et al*, 2018), 50 in Fragaria vesca (Wang *et al*., 2017), 64 in *Cucumis sativus* (Baloglu *et al*., 2014) and 200 in hexaploid hulless oat (Wu *et al*., 2017). One of the possible reasons for such differences might be due to the type and concentration of treatment and ploidy level variation as oat generated more bZIPs.

Gene ontology analysis revealed 24 genes were not involved in biological process, the possible reason might be attributed to lack of references genome of lowland bamboo and the highly divergent nature of the unigenes or novel genes that perform species specific functions (Muller *et al*., 2017). Among the annotated bZIP genes of lowland bamboo in the biological process, 15 genes were involved in stress response. Metabolic pathway analysis of lowland bamboo bZIP genes have generated only two pathway categories, environmental information processing and genetic information processing, which are believed to have strong association to plant response to abiotic stress and adaptation (Wang *et al*., 2018). From such analysis, it is easily understood that bZIPs genes were active participants in plants response to stress. Transgenic studies focusing on a bZIPs role in plants' stress response has confirmed such claims. Different members of the plant bZIP transcription factors' significant role in abiotic stress tolerances and adaptation have

been confirmed in transgenic *Arabidopsis* and rice. Such as *ABF2* conferred drought tolerances in *Arabidopsis* (Kim *et al*., 2004), *GmbZIP78, GmbZIP44* and *GmbZIP62* enabled salinity tolerance in *Arabidopsis* (Liao *et al*., 2008a), OsABI5 enhanced salt tolerance in rice (Zou *et al*., 2008). A novel *MHTGA2* bZIP gene from *Malus Hupehensis* increased salt tolerances in transgenic tobacco (Zhang *et al*., 2012). Rice group A bZIP proteins, such as *OsbZIP23* and *OsbZIP46*, also play crucial roles in ABA signaling and act as positive regulators under drought stress (Tang *et al*., 2012). Over-expression of exogenous and endogenous *AREB/ABF* orthologs in cotton substantially increased drought tolerance through stomatal regulation (Kerr *et al*., 2017). The cis-elements and expression pattern analysis of SibZIP genes indicated that SibZIPs were widely involved in responses to abiotic stresses (Wang *et al*., 2018).

Differential expression analysis also revealed that among the total 162 identified genes of bZIPs, 149 were differentially expressed (99 up-regulated while 50 are down-regulated). This unveils 61% of the identified genes were stress responsive since their expression increased due to drought and salt stress. This supports the belief that bZIP gene families are highly linked to stress response. Most importantly bZIPs have the highest number of both differentially expressed as well as a functionally annotated number of genes than any other explored transcript factor gene families of lowland bamboo. As plant stress tolerances are regulated by a combined effect of many genes, and further studies are needed to identify the complex regulatory networks of the bZIP genes in lowland bamboo. Phylogenetic analysis revealed that bZIPs of lowland bamboo has more cluster groups (14) as compared to six in cucumber (Baloglu *et al*., 2014), seven in sorghum (Wang *et al*., 2011), 10 in *Arabidopsis* (Jakoby *et al*., 2002). Clustering of bZIPs genes in to 14 groups might be attributed to hexaploid nature and reproductive biology of the species.

CHAPTER 5

5. Extent of genetic diversity and population structure of Ethiopian highland bamboo (*Arundinaria alpina* K. Schum) as revealed by SSR markers

5.1 Materials and Methods

5.1.1 Plant materials and sample collections

Young leaf samples from 150 Ethiopian highland bamboos, representing 15 populations (10 samples from each population) were separately collected and gently dried using silica gel. Sample collection was conducted with the aim of covering major highland bamboo harboring areas of the country (**Figure 12 and Table 5**).

Table 5: Sample collection areas with their respective Latitude, Longitude and Altitude

No	Place	N (Latitude)	E (Longitude)	Altitude (Masl)	Zone/Region
1	Agaro	N: 06^{0} 44’ 23.6’’	E;039^{0} 43’ 01.4’’	2583m	Jima/Oromia
2	Kofele	N: 07^{0} 05’ 45.8’’	E: 033^{0} 45’ 27.2’’	2600m	West Arsi/Oromia
3	Tikuinchnei	N: 07^{0}05’45.5’’	E: 038^{0} 45’ 27.4’’	2592m	West shewa/Oromia
4	Rira	N : 06^{0} 44’ 23.6’’	E : 039^{0} 43’ 01.4’’	2583m	Bale/Oromia
5	Yendore	N: 08^{0} 11’ 23.8’’	E: 036^{0} 26’ 33.3’’	2191m	Ilubabur/Oromia
6	Hagerslam	N: 06^{0}30’ 52.2’’	E: 038^{0}29’ 28.9’’	2598m	Sidamo/ SNPPR
7	Agena	N: 08^{0} 08’ 13.5’’	E: 038^{0}00’ 52.5’’	2201m	Guraghe/SNNPR
8	Amba-bule	N: 06^{0} 44’ 23.5’’	E: 039^{0} 43’ 01.4''	2650m	Gedeo/Oromia
9	Konta	N: 07^{0} 06’ 53.7’’	E: 036^{0} 44’53.22’’	2262 m	Dawro/SNPPR
10	Chencha	N: 06^{0}13’ 21.9’’	E: 037’ 34’ 16.1’’	2765m	Gamogofa/SNPPR
11	Masha	N: 07^{0} 35’ 12.6’’	E: 035^{0}28’ 59.8’’	2514 m	Sheka/SNPPR
12	Kosober	N: 10^{0} 56’ 04.2’’	E: 0360 56’ 54.2’’	2498m	Awi/Amhara

13	Choke	N: 10^0 33' 55.8''	E: 037^0 46' 06.1''	2968m	West Gojam/Amhara
14	Debresina	N: 09^0 51' 42.8''	E: 039^0 45' 54.7''	2595m	North Shewa/Amhara
15	Ankober	N: $09^0$42' 47.1''	E: 039^0 44' 51.6''	2488 m	North Shewa/Amhara

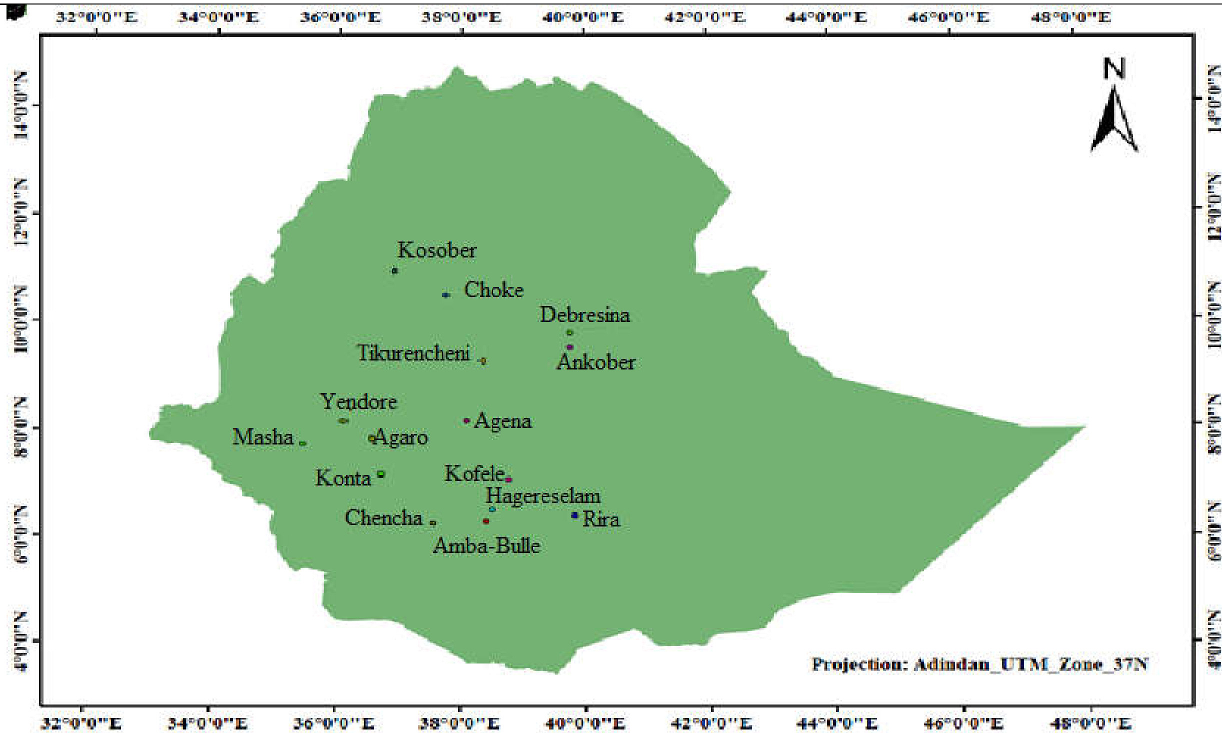

Figure 12: Map of Ethiopia showing sample collection areas

5.1.2 Genomic DNA extraction

Total genomic DNA was extracted from 50 mg 0f the silica gel dried leaves using a modified CTAB protocol as described by Doyle (1987). Quality and quantity of the extracted DNA were checked using 1% agarose gel electrophoresis and Nanodrop spectrophotometer (ND-8000, Thermo Scientific) respectively.

5.1.3 SSR marker development using reduced -representation genome sequencing and transferability

After construction of a genomic DNA library, shotgun sequencing was performed using the Illumina sequencing platform (HiSeqTM 2000) according to the manufacturer's instructions (Illumina, San Diego, CA) to generate 100 bp paired-end reads. After trimming to remove primer sequences, the short sequence reads were then assembled using SOAP *denovo* software (Zhang *et al*., 2011).

After assembly of reduced - representation genome sequences, Primer Premier 5 software (Tu *et al*., 2011) was used to design SSR primers. Then, 35 SSR primers were selected and synthesized. After checking for transferability of the primers, 10 primers were found to be transferable but only 7 primers were used for highland bamboo genotyping. Thirty SSR primers from Moso bamboo were directly selected from literature and checked for transferability but only 9 primers were found to be transferable. After screening and procedure optimization a total of 16 SSRs primer pairs (9 from Moso bamboo and 7 from Ethiopian lowland bamboo) with clear and reproducible bands were selected and used to investigate the within and among population genetic diversity and structure of highland bamboo (**Table 6**).

Table 6: List of SSR primers used for the study

No	Marker ID	Repeat Motif	FPr (5'-3')	Tm(°C)	Size(bp)	RPr (5'-3')	Tm(°C)	Size(bp)	PSize(bp)
1	*PhE842*	(CT)9	GAGGCTCCTTGGACATCACC	60.107	20	CCGACGAATAAGGCAGGCTT	60.46	20	202
2	*PhE195*	(TCC)5	CGAAGTGGAAGAGGTCGTAG	57.454	20	CTTTCCCTTGCCTCCCTTC	57.73	19	237
3	*PhE453*	(AGAGG)6	CATTCTGTTGATGGCGCTAT	56.278	20	CAGGGAGAACGACCAACTAC	57.64	20	201
4	*PhE82*	(GA)21	GGAGCGCAAATCCCATAAAG	57.218	20	CTTGCTTCGTTGCCAGTATC	57.19	20	243
5	*PhE973*	(GA)14	TTTGGGAGAGGGATTTTGCT	57.001	20	AACTCAGTGCATCAGATCGT	56.95	20	205
6	*PhE317*	(TC)10	ACTGATATTCACCCTGCAGT	56.219	20	ACAGTGGCGAAGATGAAGAT	56.93	20	212
7	*PhE196*	(AG)9	GGCTGCATAGTTCAAAGGAC	56.786	20	TGAGCGAACTACCCAAACTT	57.08	20	218
8	*PhE734*	(TC)6	TTACAATTTGAGGGCCCTGT	57.009	20	CATCTGGGTGCTGTTCTAGT	56.93	20	206
9	*PhE360*	(CT)8	CGAGGAGCCACTGATCTCAC	59.898	20	TCTCTTCCATCCCGAACCCT	59.96	20	200
10	*Oxa1*	(CCT)5	GCCTCCTTGAGATCCTCCTT	59.778	20	TGCAGCAGCAAGAACAGC	60.01	18	202
11	*Oxa2*	(GCG)6	TTTGGCGTAATCCCTGTAGC	60.096	20	TACCACAGCAGCAGCAACAC	61.09	20	213
12	*Oxa3*	(GCC)6	GACAGAAGCGGAAGTTGGAC	59.851	20	GAGAAGCAGCAGTGGAGAGC	60.44	20	190
13	*Oxa4*	(TCG)6	GAGGGACTGGATGATGGTGT	59.774	20	GAACGAGCCGTTCCAAATAG	59.71	20	202
14	*Oxa5*	(GT)6	CCCTGATGGTAGAAACACCG	60.366	20	AAAAATCCAAATGAGCATCAA	57.24	21	214
15	*Oxa6*	(GGC)6	TCGTTCTACATCCCGAGGTC	60.073	20	ACTGACCTGAACCGAACACC	60.01	20	206
16	*Oxa7*	(CTC)6	CTGTGACTGTGGATTGGTGG	59.997	20	CCTCTGACGCTGGAGCTG	60.88	18	200

Key: Markers from 1-9 were obtained from Chinese Moso bamboo and from 10-16 were obtained from Ethiopian lowland bamboo.

5.1.4 Polymerase chain reaction (PCR)

PCR amplification was done in a 12.5 µl reaction mixture (Volume) containing 2 µl of genomic DNA, 1 µl of each of the two primers (at a concentration of 10mM), 2.25 µl Nuclease free H_2o and 6.25 µl master mix. The temperature profile used during the amplification consisted of 5 minutes preheating at 94°C followed by 35 cycles of denaturation at 94°C for 30s, annealing at 54°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 10 min.

5.1.5 Agarose gel electrophoresis and SSR alleles scoring

Ten µl of PCR amplified products were loaded to 3% electrophoretic grade agarose gel with gelRed enabling to visualize DNA fragments. The gel was run at 130 V for 3 hours in 1X TAE buffer on the standard horizontal electrophoresis. A 50/100 bp Biolabs DNA ladder was used as a standard DNA molecular size. Then fragments were photographed using Altay UVP gel documentation system. Based on the expected PCR product size of each primer size, the clear bands were identified as alleles for the SSR loci. Differently sized amplified bands were scored as heterozygote alleles and vice versa. The bands were recorded as (202, 202) to represent homozygous genotypes and (202,197 or 202, 207) to indicate the heterozygous genotypes and'?' was used to denote missing data.

5.1.6 Genotypic data analysis

The number of alleles per locus, expected and observed heterozygosity, Polymorphic Information Content (PIC) and *F* statistics such as genetic differentiation (F_{st}), Wrights fixation index (F_{is}), total inbreeding coefficient (F_{it}), Nei genetic distance and identity along with Analysis of Molecular Variance (AMOVA) and principal components analysis (PcoA) for the

15 populations of Ethiopian highland bamboo were conducted using GenAlEx 6.502 (Peakall and Smouse, 2012).

Based on their computed pairwise genetic distance value, a dendrogram was drawn for the 150 highland bamboo by using Darwin V6 software (Perrier and Jacquemoud-Collet, 2006). UPGMA and NJ for the 15 highland bamboo groups was drawn by using Poptree2 (http://www.ualberta.ca/~fyeh/fyeh) program.

Population structure was inferred via the STRUCTURE v. 2.3 models (Pritchard *et al*., 2000). Three replications were run with a burn-in period of 150,000 and 150,000 repeats of the MCMC method for K value 1 to 15. The optimal number of subpopulations (K) was determined using the ΔK approach as described by Evanno *et al*. (2005) using STRUCTURE HARVESTER v0.6.8. (Earl and Holdt, 2011).

5.2 Results

5.2.1 SSR genotyping and extent of genetic diversity

From the 150 Ethiopian highland bamboo individuals, a total of 49 alleles, ranging from 2 (primers Phe453 and Oxa5) and to 4 (primers Phe734, Phe317 and Oxa1) with average of 3.06 alleles per locus were genotyped (**Table 7**). Thirteen of the sixteen markers covered two third of the total major allele frequencies. The studied loci revealed that major variation existed between observed heterozygosity and expected heterozygosity implying a significant departure from HWE across populations.

Table 7: Major allele frequency, number of alleles, observed, expected and unbiased heterozygosity and fixation index and PIC of 16 loci.

Marker	N	Major allele freq	allele No	I	Ho	He	uHe	F	**PIC**
Phe842	9.333	0.770	3	0.527	0.215	0.306	0.324	0.217	0.875
Phe195	9.667	0.546	3	0.908	0.788	0.552	0.582	-0.421	0.875
Phe453	10.000	0.500	2	0.693	1.000	0.500	0.526	-1.000	0.875
Phe82	9.533	0.775	3	0.408	0.056	0.262	0.276	0.812	0.875
Phe973	9.800	0.717	3	0.554	0.553	0.361	0.381	-0.451	0.812
Phe317	9.733	0.679	4	0.714	0.013	0.444	0.468	0.972	0.937
Phe196	9.867	0.700	3	0.559	0.000	0.338	0.356	1.000	1.000
Phe734	9.800	0.553	4	0.904	0.027	0.544	0.573	0.952	0.937
Phe360	9.800	0.712	3	0.715	0.000	0.427	0.450	1.000	1.000
Oxa1	9.600	0.504	4	0.922	0.826	0.564	0.595	-0.464	0.937
Oxa2	9.800	0.700	3	0.639	0.000	0.404	0.425	1.000	0.937
Oxa3	9.533	0.703	3	0.557	0.007	0.366	0.386	0.986	0.93 7
Oxa4	9.333	0.893	3	0.323	0.000	0.177	0.188	1.000	0.937
Oxa5	9.533	0.615	2	0.656	0.701	0.464	0.490	-0.511	0.937
Oxa6	10.000	0.713	3	0.757	0.000	0.439	0.462	1.000	0.937
Oxa7	9.667	0.863	3	0.396	0.000	0.231	0.244	1.000	**0.920**
Mean	9.688	0.684	3.067	0.640	0.262	0.399	0.420	0.417	**PIC**

Key: N (sample size), I (information Index), Ho (observed heterozygosity), He (Expected heterozygosity), uHe (Unbiased expected heterozygosity), F(Fixation index).

The actual number of alleles (Na), effective number of alleles (Ne), observed heterozygosity (Ho) and expected heterozygosity (He), Fixation index (Fst) and Polymorphic information content (PIC) across populations are presented at **Table 8**. The actual number of alleles for individual populations varied from 2.063 (Agaro) to 2.563 (Debresina and Rira). The effective

number of alleles for individual populations varied from 1.587 (Agaro) to 2.046 (Debresina), with mean Na = 2.366 and Ne = 0.179. The Ho values ranged from 0.198 (Kosober) to 0.353 (Masha). Debresina had the highest genetic diversity (He = 0.469; I= 0.755) while Agaro had the lowest genetic diversity (He = 0.333 ; 0.516). Fst value which tells us the extent of genetic differentiation among highland bamboo populations ranged from (0.273) Masha to (0.577) Kosober. The result revealed the presences of moderate genetic diversity within highland bamboo populations. The polymorphic information content scored in all the markers used is greater than 0.5 (0.812 to 1) revealing informativeness of the markers.

Table 8: Genetic diversity indices for the 15 populations used in the present study.

No	Pop	N	Na	Ne	Ho	He	I	Fst	PIC
1	***KOS***	9.625	2.313	1.698	0.198	0.369	0.601	0.577	0.875
2	***AM***	9.25	2.375	1.747	0.255	0.385	0.629	0.42	0.875
3	***AG***	9.813	2.438	1.814	0.247	0.385	0.635	0.466	0.875
4	***CH***	9.875	2.25	1.743	0.248	0.372	0.594	0.44	0.875
5	***KOF***	9.75	2.438	1.701	0.219	0.358	0.602	0.351	0.812
6	***AGA***	9.688	2.063	1.587	0.203	0.333	0.516	0.446	0.937
7	***CHO***	9.875	2.375	1.699	0.266	0.372	0.608	0.432	1.000
8	***KON***	9.688	2.375	1.759	0.212	0.385	0.627	0.52	0.937
9	***RI***	9.875	2.563	1.928	0.217	0.454	0.733	0.542	1.000
10	***ANK***	9.625	2.313	1.773	0.311	0.4	0.628	0.316	0.937
11	***DEB***	9.688	2.563	2.046	0.278	0.469	0.755	0.426	0.937
12	***MAS***	9.625	2.375	1.904	0.353	0.443	0.693	0.273	0.93 7
13	***TIK***	9.75	2.438	1.806	0.329	0.41	0.657	0.3	0.937
14	***HAG***	9.5	2.25	1.814	0.299	0.418	0.647	0.369	0.937
15	***YEN***	9.688	2.375	1.833	0.288	0.426	0.671	0.374	0.937
	Mean	9.687	2.366	0.179	**0.261**	**0.398**	**0.639**	**0.416**	**0.920**

Key 1: N (Sample size), Na (Actual number of alleles), Ne (Effective number of Alleles), Ho (Observed Heterozygosity), He (Expected Heterozygosity), I (Shannon diversity index), Fst (Fixation Index), PIC (Polymorphic Information Content).

Key 2: KOS: Kosober, AM: Amba, AG= Agena, CH: Chencha, KOF: Kofele, AGA: Agaro, CHO: Choke, KON: Konta, RI: Rira, ANK: Ankober, DEB: Debresina, MAS: Masha, TIK: Tikurichnei, HAG: Hagereselam, YEN: Yendore.

5.2.2 Genetic distance and identity among the fifteen highland bamboo populations of Ethiopia

For each pair of the fifteen highland bamboo populations' genetic identity were determined using Nei's (1972). The highest genetic identity (0.980) was observed between Kosober and Konta and Agaro and Konta. While the lowest genetic identity (0.864) was observed between Chencha and Debresina populations (Table **9**). Based on Nei-Li's similarity index, high similarity rate was observed suggesting a close relationship among populations.

Table 9: Nei's Unbiased measures of pair wise genetic identity in the 15 Ethiopian highland bamboo populations.

KOS	AM	AG	CH	KOF	AGA	CHO	KON	RI	ANK	DEB	MAS	TIK	HAG	YEN	
1.000															KOS
0.974	1.000														AM
0.924	0.918	1.000													AG
0.950	0.956	0.960	1.000												CH
0.952	0.961	0.931	0.940	1.000											KOF
0.966	0.953	0.929	0.952	0.948	1.000										AGA
0.967	0.962	0.942	0.958	0.967	0.958	1.000									CHO
0.980	0.956	0.933	0.952	0.944	0.980	0.962	1.000								KON
0.952	0.916	0.927	0.917	0.905	0.952	0.932	0.971	1.000							RI
0.953	0.941	0.934	0.941	0.942	0.946	0.968	0.965	0.938	1.000						ANK
0.877	0.873	0.904	0.864	0.900	0.876	0.906	0.892	0.911	0.927	1.000					DEB
0.914	0.892	0.904	0.895	0.897	0.901	0.940	0.918	0.928	0.932	0.935	1.000				MAS
0.930	0.919	0.884	0.898	0.916	0.890	0.946	0.908	0.895	0.939	0.927	0.942	1.000			TIK
0.915	0.897	0.892	0.868	0.912	0.889	0.940	0.901	0.915	0.924	0.949	0.948	0.970	1.000		HAG
0.939	0.920	0.883	0.890	0.915	0.914	0.941	0.940	0.934	0.956	0.939	0.947	0.963	0.961	1.000	YEN

Table 10: Nei's unbiased pair wise measures of genetic distance in the 15 highland bamboo populations.

KOS	AM	AG	CH	KOF	AGA	CHO	KON	RI	ANK	DEB	MAS	TIK	HAG	YEN	
0.000															KOS
0.027	0.000														AM
0.063	0.060	0.000													AG
0.049	0.039	0.031	0.000												CH
0.048	0.034	0.059	0.057	0.000											KOF
0.033	0.038	0.056	0.039	0.048	0.000										AGA
0.029	0.030	0.046	0.039	0.031	0.038	0.000									CHO
0.018	0.034	0.052	0.041	0.050	0.019	0.031	0.000								KON
0.036	0.058	0.050	0.059	0.071	0.041	0.046	0.024	0.000							RI
0.042	0.049	0.053	0.051	0.050	0.046	0.025	0.030	0.042	0.000						ANK
0.080	0.085	0.065	0.091	0.075	0.087	0.059	0.069	0.047	0.042	0.000					DEB
0.065	0.078	0.068	0.078	0.076	0.076	0.044	0.060	0.042	0.042	0.033	0.000				MAS
0.057	0.062	0.079	0.074	0.065	0.084	0.039	0.068	0.065	0.041	0.043	0.036	0.000			TIK
0.066	0.076	0.077	0.095	0.071	0.085	0.044	0.072	0.052	0.050	0.027	0.029	0.020	0.000		HAG
0.051	0.064	0.080	0.081	0.068	0.067	0.044	0.047	0.040	0.028	0.032	0.031	0.026	0.025	0.000	YEN

The highest genetic distance (0.091) was observed between Chencha and Debresina populations. While the lowest genetic distances (0.018) was observed between Kosober and Konta populations (**Table 10**).

5.2.3 Population structure, principal component and cluster analysis

STRUCTURE algorithm was applied to measure population structure and degree of admixture. The highest log-likelihood score was attained for K=9 (Figure **13A**). As shown in (**Figure 13B**), population structure analysis merged the 15 populations in to 9 distinct classes (populations). A

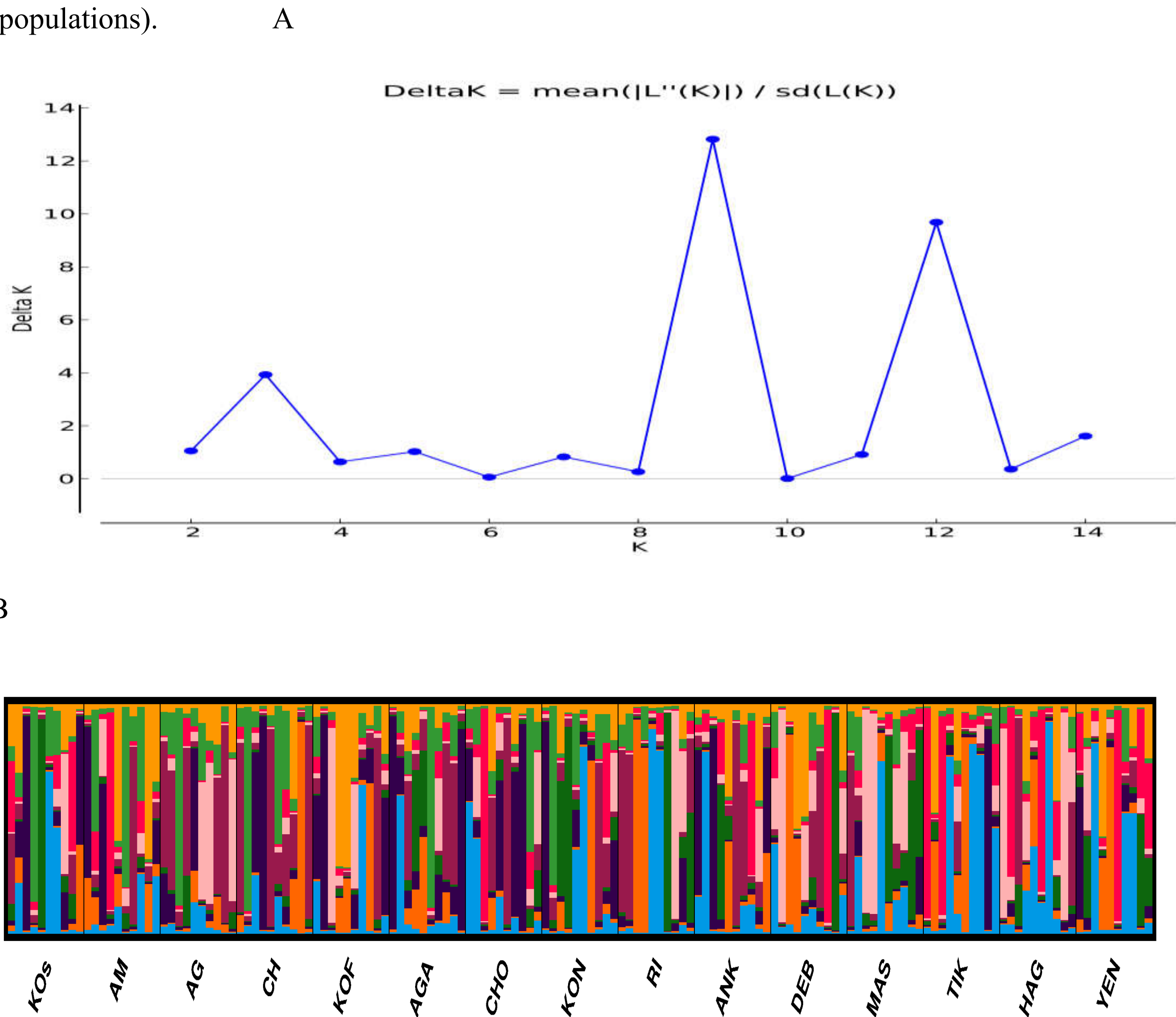

Figure 13: Structure bar graph of highland bamboo groups inferred at K = 9 based on SSR data.The relationship between ΔK and K showing the highest peak at K = 9.

Although the structure analysis (bar plot) revealed 9 distinct groups of the entire 15 populations members of a population were not grouped together under a single cluster, instead placed under different clusters with individuals from other populations (**Figure 14**).

In PCoA, the first three principal coordinate accounted for 25.36 % of the total variation in which the first principal component accounted for 9.85% of the total variation, while the second and third principal coordinates accounted for 8.07% and 7.44 % of the total molecular variances respectively.

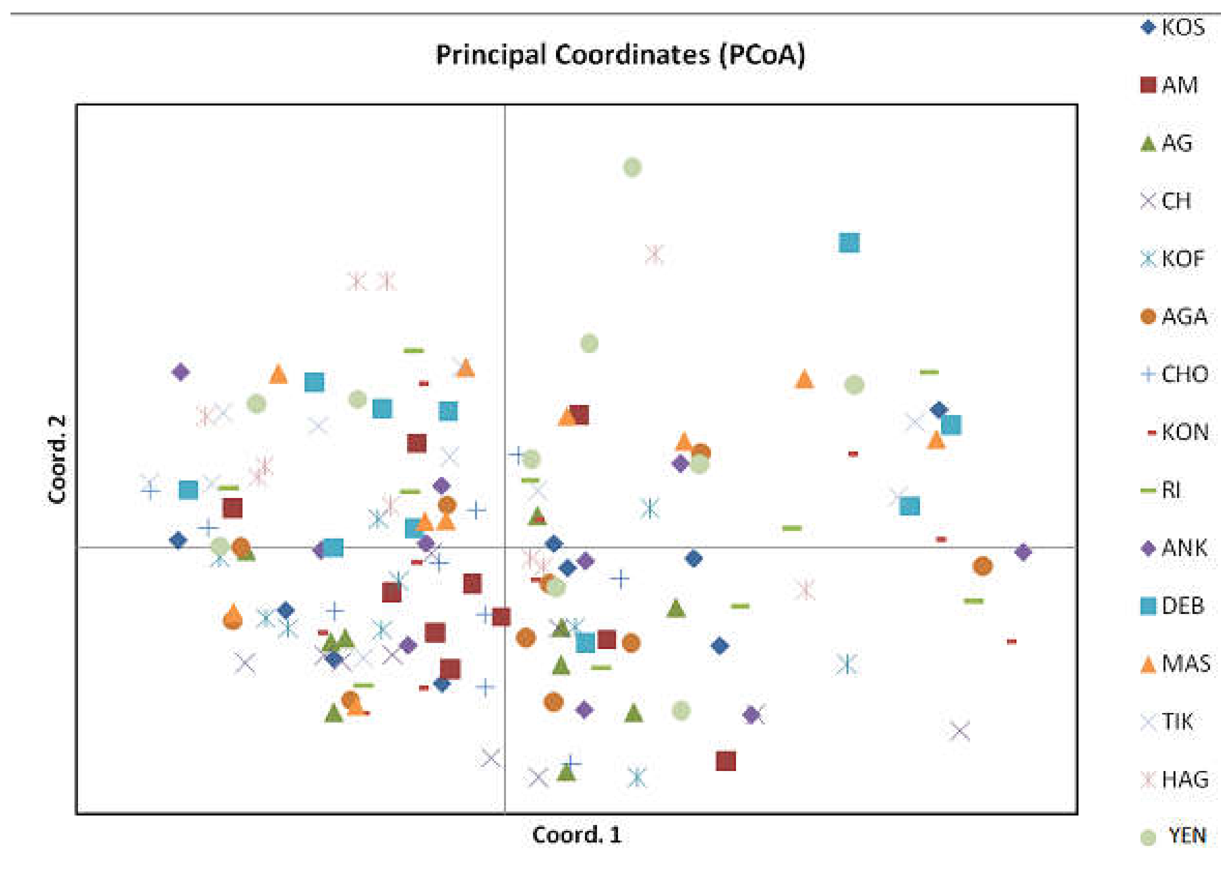

Figure 14: Principal coordinates analysis (PCoA) bi-plot showing the clustering pattern of 150 samples representing 15 populations. Samples coded with the same symbol and colors belong to the same population.

Neighbor joining clustering of the 150 highland bamboo individuals resulted in to 9 groups in (**Figure 15**). However, individuals of the same population were not exclusively clustered together, instead dispersed over different clusters (branches) reflecting high level of genetic admixture.

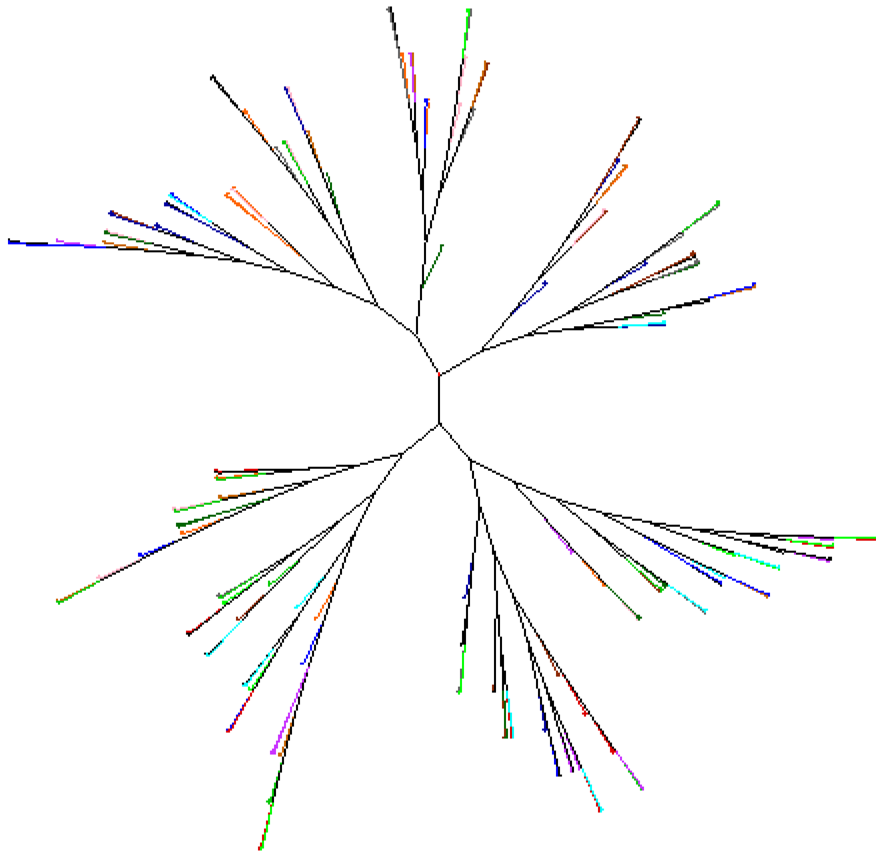

Figure 15: Neighbor-joining tree generated based on 150 highland bamboo individuals.

UPGMA based population phylogeny clustered the 15 populations into 2 main clusters (1 and 2) with 5 sub-clusters (A,B,C,D, C and E). As presented in **Figure 16,** populations were not grouped on the basis of the proximity of their geographical collection.

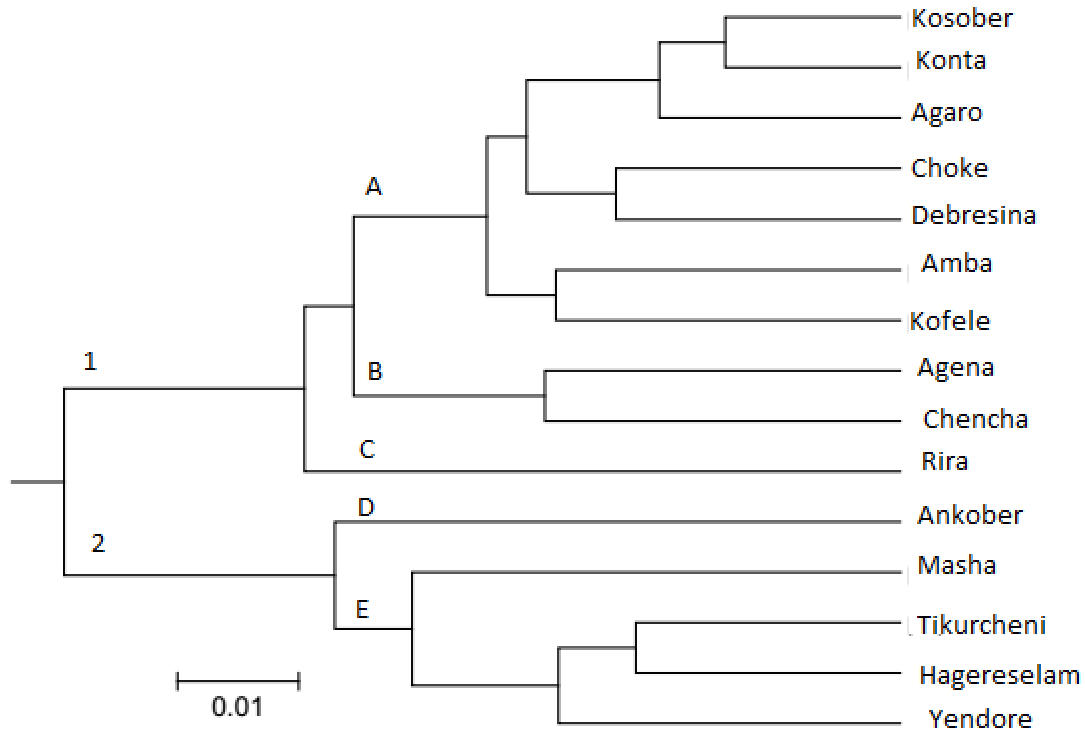

Figure 16: Un-weighted pair-group method with arithmetic mean (UPGMA) Dendrogram showing genetic relationships among the 15 populations considered based on Nei's unbiased genetic distance.

Populations based Neighbor joining clustering grouped highland bamboo populations in to three main groups. Accordingly, populations from Debresina, Tikurcheni, Hagreselam, Masha and Yendore were clustered together under (Cluster I). Choke, Kosober, Amba, Kofele, Chencha, Agaro and Rira were clustered under (Cluster III). But one population, Ankober has become monophyletic and grouped under (Custer II) (**Figure 17**).

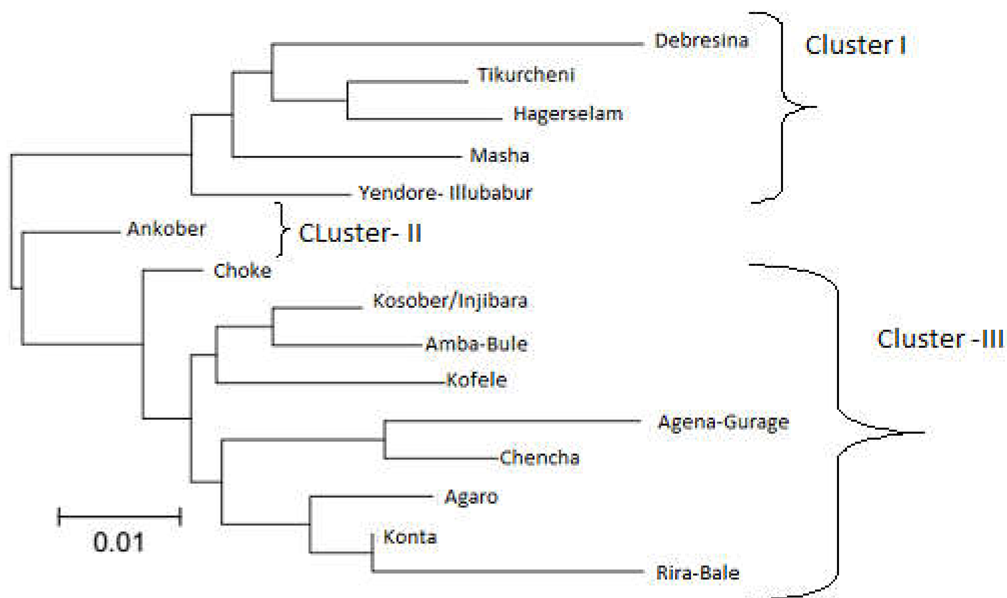

Figure 17: Dendrogram showing the genetic relationships among 15 populations using Neighbor joining analysis.

5.2.4 Genetic differentiation and variance analysis of highland bamboo in Ethiopia

Paired comparisons among populations based on locus by locus analysis for the 16 microsatellite loci revealed that Fst ranged from 0.000 at locus Phe453 to 0.265 at locus Phe82. The value of F-statistic analysis showed that there are no any loci that showed complete differentiation as there is no loci with Fst value one. According to F-statistics majority of the loci showed weak genetic differentiation (**Table 11**).

Table 11: F-statistics for the 16 loci across the study populations

Locus name	Fis	Fit	Fst
Phe842	0.298	0.432	0.191
Phe195	-0.428	-0.316	0.078
Phe453	-1.000	-1.000	0.000
Phe82	0.785	0.842	0.265
Phe973	-0.531	-0.339	0.126
Phe317	0.970	0.972	0.078
Phe196	1.000	1.000	0.165
Phe734	0.951	0.955	0.088
Phe360	1.000	1.000	0.043
Oxa1	-0.464	-0.394	0.048
Oxa2	1.000	1.000	0.130
Oxa3	0.982	0.984	0.148
Oxa4	1.000	1.000	0.100
Oxa5	-0.511	-0.480	0.020
Oxa6	1.000	1.000	0.014
Oxa7	1.000	1.000	0.030
Mean	0.441	0.479	0.095
SE	0.186	0.179	0.018

Fis - inbreeding coefficient among individuals within population, Fit- fixation index among individuals within total data set, Fst-genetic differentiation among populations.

Analysis of molecular variance (AMOVA) revealed that the highest genetic variability (53%) was accounted to within individuals than among populations (2%) (**Table 12**).

Table 12: Summary of AMOVA for the 15 Ethiopian highland bamboo populations based on 16 SSR markers.

Source	DF	SS	MS	Est. Var.	%age	Fst	P value
Among Pops	14	96.707	6.908	0.074	2%	0.019	0.001
Among Indiv	135	734.05	5.437	1.702	45%		
Within Indiv	150	305	2.033	2.033	53%		
Total	299	1135.76	14.378			0.533	

Df= Degrees of freedom; SS= Sum of squares; MS= Mean Squares; Fst= Fixation index

5.3 Discussion

Most bamboo populations are established through vegetative propagations. Sometimes a population might be of a single rhizome clonally extended to a vast area. Hence, all individuals of a population might have similar or nearly similar genetic makeup (Stern, 2004). The genetic similarity might result in total loss of the population if a certain disease or pest arises. Flowering creates the opportunity of gene mix up in the next generation since seeds can have more genetic variability. Bamboo is an out-crossing wind pollinated plant where there is high gene random mix up although flowering is an extremely rare event or ones in life. As bamboos are mostly multiplied clonally, a single plant can form huge populations over time (Miyazaki *et al*, 2009). This may follow a deficit of genetic variation (Wong, 2004).

The SSR markers used in this study contained di-, tri and penta nucleotide repeats. Di- and Tri-nucleotide repeat motifs were abundant. Studies have shown that di and tri repeat SSRs are the most commonly used motifs in molecular genetic studies (Selkoe and Toonen, 2006).

By employing different markers, the level of genetic diversity for different bamboo species has been conducted in different countries. Using 42 SSR markers (34 rice and 8 sugarcane SSR primers) Sharma *et al*., (2008) reported limited genetic variation in *Dendrocalamus hamiltonii* (H= 0.250). Reyes *et al*., (2015) observed better genetic variation (H= 0.253 and Fst= 0.483) in *Bambusa blumeana* and the same is true for Meena *et al*., 2018, (H= 0.265 and I = 0.285) in *Dendrocalamus hamiltonii*.

The within-population variation was detected for 7 *Dendrocalamus giganteus* populations using 7 ISSR markers (H = 0.042, PIC = 11.33%) (Tian *et al*., 2012). Yang *et al*., (2012) also reported (H = 0.164, I= 0.249, and PIC = 48%) using 10 ISSR markers for 12 *Dendrocalamus membranaceus* populations. For 18 Moso bamboo populations Zhang *et al*., (2007) observed a Shannon's information index (I) value of 0.377 using RAPD markers. Lai and Hsiao (1997) observed a very limited genetic variation in Moso bamboo from Taiwan based on 13 RAPD markers and three microsatellites. Ruan *et al*., (2008) observed the percentage of polymorphic content (PIC) of 38% based on AFLP and PIC of 39.9% on ISSR markers from 17 Moso bamboo provenances. Jiang *et al*., (2012) presented (I= 0.532, H=4.40) based on 20 SSR markers in Moso bamboo. Dong *et al*., (2012) observed (H=4.32) in *Dendrocalamus sinicus* using 16 SSR markers. Recently Jiang *et al*., (2017) has presented (H = 0.32, I = 0.499, PIC = 74.4%) was revealed within the 34 Moso bamboo populations using 20 SSR markers. As reported in Atigala *et al*., (2017) Sri lanka's *Kuruna debilis* resulted in to (H= 0.758 , Fst= 0.113) using 12 SSR markers.

In contrary, moderate genetic diversity (H= 0.398, Fst= 0.019, I= 0.639, PIC = 92%) was revealed within the 15 Ethiopian highland bamboo populations. Such differences might be raised due to the markers used, sampling strategy and sample number.

In all studied loci, there are differences between observed heterozygosity and expected heterozygosity showing heterozygosity that led to a significant departure from HWE across populations. Such heterozygosity is expected in out-crossing species that maintain their heterozygosity through vegetative propagation, or if other factors such as natural and artificial selection pressures favor heterozygosity (Morin *et al*., 2009).

Plants life form and the breeding system had highly large influences on the genetic diversity and predominately outcrossing woody species had more genetic diversity than predominately herbaceous species (Hamrick and Godt, 1996). Conversely, high genetic diversity has been reported in some clonal plants using molecular markers (Chen *et al*., 2010; De Witte *et al*., 2012). Regardless of the breeding system, the population history habitat characteristics and geographic distribution of populations have a significant impact on the genetic diversity of clonal plants (Jiang *et al*., 2017). Highland bamboo, as is common to other bamboos, is characterized by a prolonged vegetative phase, long flowering intervals (10–120 years), and gregarious flowering (Sertse *et al*., 2011; Isagi *et al*., 2016). So, with such long vegetative phase without the exchange of genetic material through pollen transfer, moderateness of the genetic diversity is expected.

On the basis of both Nei's genetic identity and genetic distances, higher genetic identity but very lower genetic distance was observed among highland bamboo populations. This tells us that on the basis of their genetics the populations are highly closer despite their differences in their geographic origin of collection. This strongly supports the presences of the genetic mix that might arise as a result of using vegetative culms for propagating the plant.

Both population structure and individual phylogeny merged the 15 populations into 9. However, individuals from the same population were not clustered together, instead dispersed to other populations. This shows the presences of higher genetic admixtures between individuals. The Principal component analysis also revealed highly dispersed individuals still showing the presences of genetic admixture. UPGMA clustered the 15 populations into 2 groups while NJ clustered in to 3 groups. Although the clustering in both cases is not in line with the origin of collection, the clustering of NJ is better in terms of the geographical proximity of populations than UPGMA. In NJ clustering Ankober independently formed one cluster while in UPGMA it again independently forms a single sub-cluster. The possible reason for Ankober to be monophyletic might be due to its protected and inaccessible location. As bamboo is reproduced mainly through vegetative propagation using culm cutting or rhizome, transferring such materials from Ankober to other areas might be difficult when compared to locations of other populations.

Hierarchical analysis of molecular variance (AMOVA) showed very low population differentiation with only 2 % of the total variation accounted for variation among populations. Such conditions are common particularly for vegetatively propagating perennial and outcrossing species which are heterozygous and retain high level of genetic variation within populations

(Hamrick and Godt 1989). Generally, the level of genetic differentiation of overall populations was very low (Fst = 0.019). This differentiation was by far lower than those observed in other bamboos. For instances, as reported in Tian *et al.,* (2012) *Dendrocalamus giganteus* (Fst = 0.847) and Yang *et al.,* (2012) in *Dendrocalamus membranaceus* (Fst = 0.252). Fst value is in a range of 0.05 to 0.25 shows moderate differentiation among populations and Fst > 0.25 shows higher differentiation (Wright, 1978).

6. CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study has investigated and presented the first comprehensive global transcriptome profiling of lowland bamboo under drought and salt stress conditions using the Illumina sequencing platform. The study showed that multiple genes, transcript factors and pathways are involved in drought and salt response. Differentially expressed genes analyses have suggested the stress responsiveness of lowland bamboo transcriptome to drought and salt stress, since 65, 471 genes were differentially expressed. The predicted 4,332 transcriptions factors (TFs) organized into 64 TFs families uncovered the most important transcript factor gene families associated with abiotic stress response. The commonly regulated 569 genes in both abiotic stresses are potential candidates for engineering plants for multiple abiotic stress tolerance and adaptation. In general, this transcriptome analysis had identified the key genes, transcription factor families, pathways that can be easily exploited for further transgenesis experiments aiming to investigate genes that are blessed in conferring abiotic stress tolerance and adaptation in plants.

The genome-wide scale analysis of the bZIP gene family members in lowland bamboo was conducted and identified a total of 162 *Oa*bZIP proteins. The study has presented valuable information pertaining to bZIP gene families role in lowland bamboo associated with abiotic stress. Both the gene ontology and metabolic pathway analysis has magnified the stress responsiveness of bZIP genes as the level of expression of 99 genes was increased in response to drought and salt stress. Those bZIP genes with the highest level of expression could serve as the foundation for further functional characterization, elucidating their specific regulatory

mechanisms and eventually could be applied for genetic improvement programs aiming to enhance plant growth and development in the face of abiotic stress.

With regard to SSR based genetic diversity analysis in Ethiopian highland bamboo populations, Debresina population showed the highest genetic diversity while Agaro had the lowest genetic diversity. In terms of population genetic differentiation, Kosober population has the highest genetic differentiation within itself, while Masha population has the lowest genetic differentiation. Both population structure and individual phylogeny merged the 15 highland bamboo populations into 9 groups, but individuals of the same population did not cluster together, rather dispersed, implying a higher level of genetic admixture. In conclusion, from genetic diversity analysis, it's understood that the genetic base of Ethiopian highland bamboo populations is neither wide nor narrow rather it is moderate. In general, the study had attempted to investigate the genetic diversity and structure of highland bamboo populations which could be used as a basis for conservation interventions, improvement programs and/or for further investigations

6.2 Recommendations and future research directions

This Ph.D. research has investigated; First, the drought and salt stress-induced RNA-seq global transcriptome profiling in Ethiopian lowland bamboo. Second, the Genome-wide analysis and expression profiling of bZIP transcript factor gene family under drought and salt stress in lowland bamboo. Third, through reduced-representation genome sequencing of Ethiopian lowland bamboo, SSR markers were developed then with those directly taken from literature from Moso bamboo transferability was checked for their polymorphism. Finally, polymorphic

markers, 9 from Chinese Moso bamboo and 7 from Ethiopian lowland bamboo were selected and used for highland bamboo genetic diversity analysis. Fourth, the extent of genetic diversity and population structure of highland bamboo populations as using SSR markers were investigated.

However, the following issues should be addressed in future research.

1. The RNA - seq transcriptome profiling has identified key drought and salt stress-responsive genes, but validating such genes through transgenesis research using model plants is an assignment for future research.

2. The bZIPs transcript factor has been identified and characterized on the basis of phylogeny and expression, but the functional characterization and elucidating their specific regulatory mechanisms are left to be addressed.

3. As Kosober population has the highest genetic differentiation, more conservation attention deserves for this population. The employed 16 SSR markers were able to moderately capture the genetic variability of highland bamboo populations. Repeating genetic diversity research with more number of SSRs markers and SNPs that are capable of capturing the entire genetic variability is very important so as to elucidate the actual genetic variability of highland bamboo populations. Highland bamboo populations have distinct morphology in Injibara/kosober, Masha and Chencha areas. So conducting genetic diversity study particularly focusing on these three areas may end up in supporting claims of such morphological variation.

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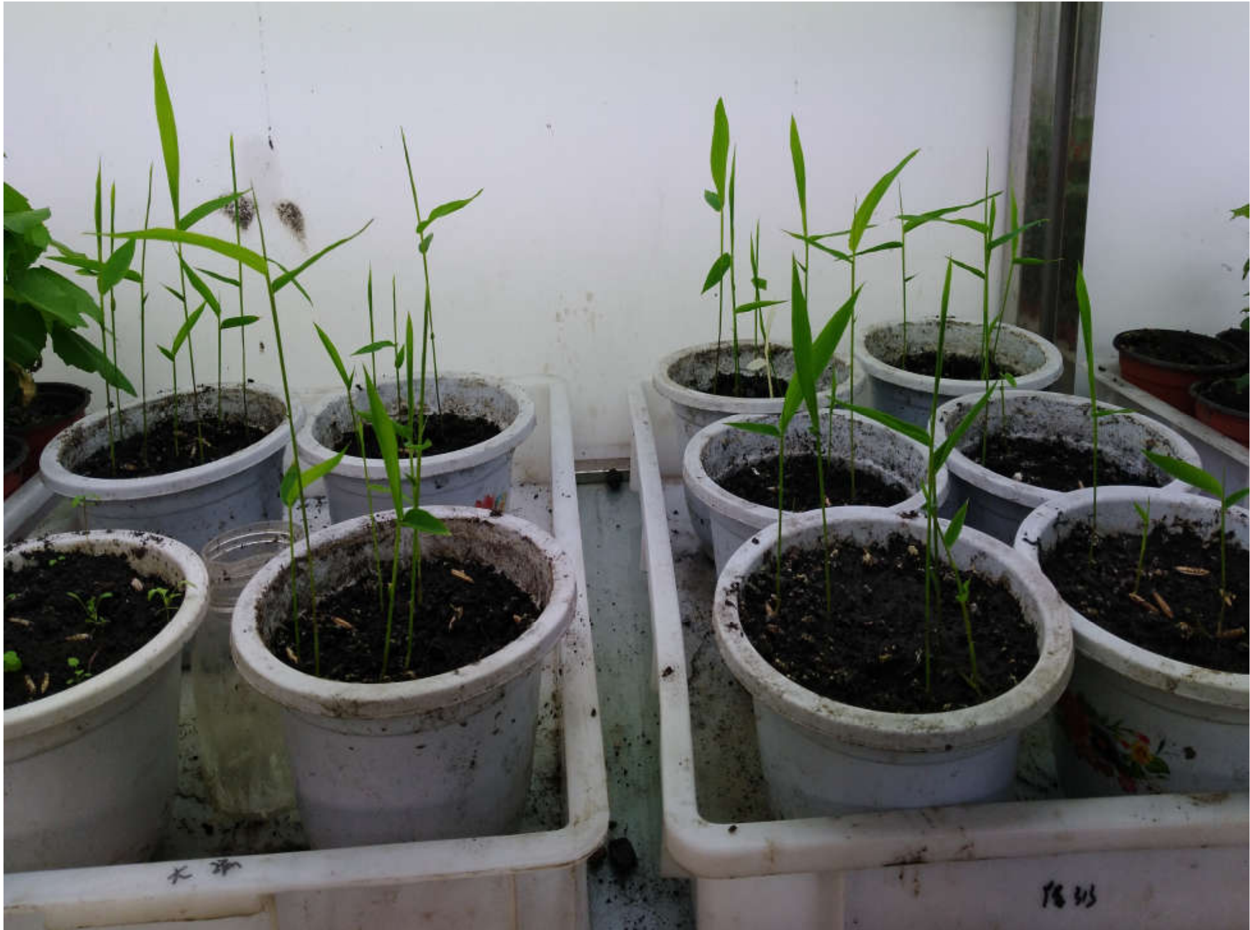
8. LIST OF APPENDICES

Appendix 1. Germinated seedlings of lowland bamboo



Appendix 2: Lowland bamboo seedlings ready for treatment

Appendix 3 qPCR primers used for validation of RNA-Seq data

Gene ID	Gene Regulation		Sequences	length	Tm	GC%	Product
TRINITY_DN122470_c0_g1_i1	up	Forward(FPr)	GGCTCCTCGACTACCAAC	18	61	52.4	97
		Reverse(RPr)	GCTCACCTACTGAGAGG	17	61	55	
TRINITY_DN120395_c1_g1_i3	up	FPr	CAACAAGGTATGTGAGGACG	20	62	45.5	112
		RPr	CTCCATAGATGTCTGCTGTCG	21	62	50	
		FPr	CTGTTACTCTACATCCAAAGAG	22	62	50	103
		RPr	GGTATCATCACTACTGCGACG	21	62	50	
TRINITY_DN143129_c3_g1_i3	up	FPr	CAGAGATCGGGTGGGAC	17	62	50	113
		RPr	TCGTATGAGCAGGTGGG	17	62	50	
		FPr	CGATGGCTTTGTTCAGGAAAC	21	62	45.5	120
		RPr	CCTGACATAGCCTCCACAC	19	62	50	
TRINITY_DN120395_c1_g1_i4	down	FPr	CAACAAGGTTTGTGAGGACG	20	62	45.5	112
		RPr	CTCCATAGATGTCTGCGCG	19	62	50	
		FPr					
		RPr					
TRINITY_DN143927_c3_g2_i8	up	FPr	GGTCACACAGCAGATGGA	18	62	50	121
		RPr	GCTGAGATGTGAGGAGACA	19	62	45.5	
TRINITY_DN149604_c14_g4_i4	down	FPr	TCTGAGGCTGTGGGCAA	17	62	58.8	108
		RPr	TCAAGAACAGTGGAGAAGACG	21	58	41.7	
TRINITY_DN128874_c0_g1_i1	down	FPr	GAGGAGTGGTATTCAAGGAAGG	22	62	50	101

		RPr	CCCATCAACATTTGCTCCAAC	21	62	45.5	
TRINITY_DN148850_c1_g2_i1	down	FPr	CTACGCCTATGATGGAACCAAG	22	62	50	112
		RPr	CAGGTGAAGAACAACTCCTACG	22	62	50	
		FPr	GAGTTGGAGCAACGGATAG	19	62	52.4	112
		RPr	GATGTTCTCGGCTGATG	17	62	50	
TRINITY_DN100003_c0_g1_i2	up	FPr	GTCCTGACATGGATGTGGAAG	21	62	47.8	99
		RPr	GGGCTTTCAGCAGTCGAAAA	20	63	47.6	
TRINITY_DN100003_c0_g1_i1	up	FPr	CCAGATATTTGAAGCCCGAGAA	22	62	45.5	100
		RPr	TCAGCCACTTGTAACGCAC	19	62	47.6	
TRINITY_DN115811_c0_g1_i1	down	FPr	GAGACTGTTGTCCTCAAGG	19	62	47.6	108
		RPr	CTTCAGGTCTATGTCGAAGG	20	62	50	
TRINITY_DN10590_c0_g1_i1	up	FPr	TGGAGACTATCACCTCAGAAGA	22	62	45.5	95
		RPr	TAGAGCACCAGCATTGACAC	20	62	50	
TRINITY_DN1058_c0_g1_i1	up	FPr	GAGTCTGACGGTGGAGGA	18	62	61.1	122
		RPr	AGCAGAGCTGGAGGAAC	18	62	55.6	
TRINITY_DN105699_c0_g2_i1	down	FPr	ATGAGTGGACAGCAACCAAG	20	62	50	104
		RPr	GTTGGGAGTGAGTGAGACAAC	21	62	50	
		FPr	GTCTCACTCACTCCCAACAC	21	62	52.4	103
		RPr	GTATTTGTGGACCAGGCACA	21	62	47.6	
TRINITY_DN105465_c0_g1_i1	up	FPr	TGCATGCAGTTGCTGTG	17	61	50	108
		RPr	AGTGATGATGCACATGAGAGA	21	61	40.9	

TRINITY_DN101328_c1_g3_i1	up	FPr	CGGAAGAACAGGTGGAGAG	19	61	57.9	121
		RPr	ACCAATTGCACTTTGTGAGG	20	61	45	
TRINITY_DN101241_c3_g1_i1	up	FPr	GAATCTGCGGGAAGTAGAGC	20	62	50	125
		RPr	TCCTGACCTATATGCTGC	18	62	45	
TRINITY_DN101051_c0_g1_i1	up	FPr	GCTCAGATGGGTACCACAAAA	21	62	45.5	100
		RPr	GGAATAAGCCTGGACCATGAA	21	62	47.6	
		FPr	CAATCCGAAAGGGCTCGAAA	20	62	47.6	97
		RPr	AGGGAAATCGAGATGGTAGAGA	22	62	45.5	
TRINITY_DN101045_c1_g2_i1	down	FPr	GTCGGCTCCACCAACAAA	18	62	55.6	88
		RPr	CAATGACAGGAGATGCCAGAG	21	62	52.4	
TRINITY_DN101015_c0_g1_i1	down	FPr	GCAACTCCGTGAAAGGAGAA	20	62	50	102
		RPr	CTCCGTGGGAAGAGAAGAGA	20	62	55	
TRINITY_DN100974_c0_g2_i1	down	FPr	TGGAGGAAAGAATAGGCAGG	20	62	47.6	141
		RPr	CTTGATCTGGCGGCTGA	17	62	52.6	
TRINITY_DN147250_c1_g1_i1	down	FPr	AAAGAACGCCAGAGGCAAG	19	62	50	147
		RPr	CAGATGGGAACCACTCCAAC	20	62	50	
TRINITY_DN135948_c0_g1_i3	up	FPr	GCGATAGCTCGTGAGAAAGA	20	61	50	104
		RPr	GGTGGAGGCAGGTTCAG	17	61	64.7	
TRINITY_DN134591_c3_g2_i3	down	FPr	AACAACCTGCTGGGAGG	17	62	55.6	79
		RPr	CGAGGGTGGAGGTGTAGA	18	62	57.9	
TRINITY_DN152985_c4_g2_i10	down	FPr	GATGGCGAAGCCTTGGAA	18	62	55.6	118

		RPr	GTCCTTGTTGACGAGCAGA	19	62	50	
TRINITY_DN35594_c0_g1_i1	down	FPr	GAGCGCTACCTCAACGAC	18	62	61.1	108
		RPr	GTACATGTGGACGAGCGA	18	62	50	
		FPr	TCGTACGACGTGCTGGA	17	62	58.8	105
		RPr	GATGAGGTGCACGGGAG	117	62	57.9	
TRINITY_DN147605_c3_g1_i2	down	FPr	CGAGACGAAACAGAGTCACAC	21	62	50	100
		RPr	CTCCGAACTCGACTTCTTCAC	21	62	50	
		FPr	CGAGCATAGTGGAGGAGGA	19	62	57.9	105
		RPr	TGTAGTTGAGCAGCGAGG	18	62	50	
TRINITY_DN156096_c6_g5_i1	down	FPr	GAAGGTCAAGGCAAAGAAACAC	22	62	43.5	123
		RPr	ACTCAGTGGAATCAGCACAC	20	62	47.6	
TRINITY_DN149358_c0_g2_i2	down	FPr	CGATGAAGAGCGGGAAGAAC	20	62	50	147
		RPr	CCATGTCCTTAAACGGAGGAA	21	62	45.5	
TRINITY_DN51686_c1_g1_i1	down	FPr	GTCTGGCTCGACAAGACAAC	20	62	50	92
		RPr	TCCTCGATATGGAGAAGGAGAC	22	62	47.8	
		FPr	GGCCTTGATAACTGGCAGAA	20	62	50	104
		RPr	CCAACCTGACACTTGCAGA	19	62	50	
TRINITY_DN152902_c2_g3_i5	down	FPr	GCTGCTGTAGGAACAACCAA	20	62	45.5	126
		RPr	GCGAGTCTGAAGCATGCAA	20	62	47.6	
TRINITY_DN82270_c0_g1_i1	down	FPr	TCAACCGAGGAAGAGAGAAGA	21	62	47.6	101
		RPr	GCCAACTCCATGGGACAA	18	62	52.6	
TRINITY_DN6202_c0_g1_i1	down	FPr	GTGCAGAGAAGTCAGGAAGG	20	62	55	96

		RPr	CAGAAACTCAAGAAGCACCA	20	62	39.1	
TRINITY_DN183844_c0_g1_i1	up	FPr	TGGAGCTAAGAAACTCGAAAGG	22	62	45.5	100
		RPr	ACAGCAGGAACACCATAAGAC	21	62	47.6	
TRINITY_DN19948_c0_g1_i1	up	FPr	CTGCGGCTACTCCAGAA	17	62	52.6	106
		RPr	GTTGAAACCCATGACCCAAAC	22	62	43.5	
TRINITY_DN219305_c0_g1_i1	up	FPr	GGCCATTTCCTCACCGAAA	19	62	50	108
		RPr	CCACTGAAGCAGCATCCAA	19	62	50	
TRINITY_DN84199_c0_g1_i1	up	FPr	CCGTGATGCCCAAGAGAAA	19	62	52.6	98
		RPr	CAGTCTCCCAACAACGAAC	19	62	50	
TRINITY_DN948_c0_g1_i1	up	FPr	AGGCCAAGGAAGCACAG	17	62	58.8	136
		RPr	CACCATCACAAGAAGAAGAAGAAG	24	62		
TRINITY_DN169111_c1_g1_i1	up	FPr	TGAGGAGGTTGAGAAGCAGA	20	62	45.5	99
		RPr	GAGTCTCCAGATTGATGGAAACA	23	62	43.5	
TRINITY_DN22970_c0_g1_i1	up	FPr	TCTACCTAACCCTCTACGGAAC	22	62	50	96
		RPr	ATGAGTTGTGGTAGGAGGAGA	21	62	47.6	

Appendix 4: qRT-PCR validation of RNA-Seq data. It tells the correlation of the fold change analyzed by RNA-Seq platform (x-axis) with data obtained using qRT- PCR (y-axis).