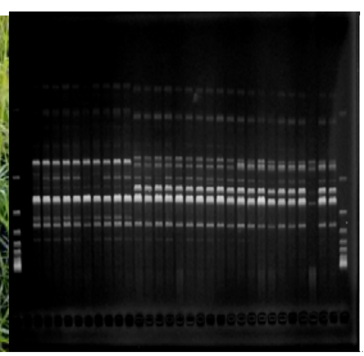
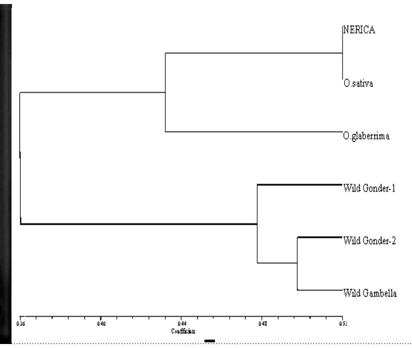
ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
DEPARTMENT OF BIOLOGY

RELATIONSHIP BETWEEN WILD RICE SPECIES OF ETHIOPIA WITH CULTIVATED RICE BASED ON ISSR MARKER

A Thesis Presented to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (Applied Genetics)

GEZAHEGN GIRMA

July, 2007

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List of Abbreviations

AFLP-Amplified Fragment Length Polymorphism

AMOVA-Analysis of Molecular Variance

CSA-Central Statistical Authority

GD- gene diversity

H- Shannon's diversity index

IRRI-International Rice Research Institute

ISSR-Inter Simple Sequence Repeat

NERICA-New Rice for Africa

NPL-number of polymorphic loci

NJ-Neighbor Joining analysis

PCO-Principal coordinated Analysis

PP (%) -percent polymorphism

RFLP-Restriction Fragment Length Polymorphism

RAPD-Random Amplified Polymorphic DNA

SNPs- Single Nucleotide Polymorphisms

UPGMA – Unweighted pair group method with arithmetic mean

Abstract

Three wild rice populations of Ethiopia (one from Gambella and two from Gonder) and three cultivated rice populations (*O. sativa* from Gonder, *O. glaberrima* from West Africa and NERICA rice (NERICA-3; cultivar developed through interspecific hybridization between *O. sativa* and *O. glaberrima*), from Gonder) were studied. Inter simple sequence repeats (ISSRs) as a molecular marker was used to assess genetic diversity both within and between species using six ISSR primers, where four of them were dinucleotides and two tetranucleotides primers. A total of 93 clear and reproducible bands were amplified from the six ISSR primers. Both UPGMA and neighbor joining trees were constructed for each individual and population using Jaccard's similarity coefficient of '0' absent and '1' present data. The trees clearly indicated six distinct groups which are based on populations of origin. The PCO analysis also recovered the UPGMA and neighbor joining trees groups, although wild Gonder-1 intermixed with wild Gonder-2. *O .glaberrima*, *O. sativa* and NERICA-3 clustered as a major group while *O. barthii* and *O. longistaminata* were clustered as the second major group. Such clustering of *O. glaberrima* with *O. sativa* and NERICA-3 is considered to be due to genetic admixture of *O. glaberrima* with *O. sativa*. The genetic diversity result generally indicated that wild rice populations were found to have higher gene diversity (0.14) than cultivated rice populations. The over all gene diversity and percent polymorphisms were found to be higher in wild rice than in cultivars (0.11). The Shannon's diversity index also confirmed the existence of higher diversity in wild rice populations of Ethiopia than cultivated species used in the present study. Furthermore, partitioning of the Shannon's diversity showed that the majority of the variations were observed among population (63%). Similarly, AMOVA demonstrate highly significant (P=0.00) genetic differences among populations within groups, among groups (cultivated and wild) and within populations. Of the total variation, 49.4% was attributable to among populations within groups, 26.4% to among groups and the least, 24.2% to within populations.

Key words: genetic diversity, wild rice populations, cultivated rice, *Oryza sativa*, *Oryza glaberrima,* NERICA, ISSR

Declaration:

I, the undersigned, here by declare that this thesis is my original work and that all sources of materials used for the thesis have been duly acknowledged.

Name:______________________________

Signature: __________________________

Date:______________________________

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By

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1. Introduction

Rice is a unique crop of great antiquity and akin to progress in human civilization (Chang, 2002). It is an annual grass in the grass family, Poaceae. Asian rice is classified as *Oryza sativa* L. and African rice as *Oryza glaberrima* Steud. (Thakur, 1975).

Rice is one of the leading cereal crops of the world and is the principal food crop of about half of the world's populations. In many regions it is eaten with every meal and provides more calories than any other single food (Stoskopf, 1985). According to Onwueme and Sinha (1991) rice can also be used in the manufacture of cosmetics and textiles, beer and wine are also made from rice.

According to Onwueme and Sinha (1991) there are 25 species in the genus *Oryza*, of these only two species are cultivated, namely *Oryza sativa* and *O.glaberrima. Oryza sativa* is the common rice grown throughout the warmer regions of the world whereas *O. glaberrima* is grown to a limited extent in the flood plains of West Africa. Only two of them offer an agriculture interest for humans (UNCTAD, 2003)

The wild relatives of rice possess certain desirable genes which, when transferred to cultivated species, enhance the agronomic characters of cultivars. Some examples from IRRI's genetic evaluation and utilization program are: useful traits such as resistance to brown plant hopper, white backed plant hopper from *Oryza eichingeri* Peter, resistance to brown plant hoper and tolerance for drought from *O. australiensis* Domin, resistance to brown plant hopper, white backed plant hopper from *O. minuta* J.S.Presl ex C.B.Presel., resistance to brown plant hopper, bacterial blight and bacterial leaf streak from *O.punctata* Kotschrex.Steud (Villegas, 1990).

Villegas (1990) also indicated that introgression of genes from wild relatives to cultivated species will enhance the genetic variation essential to rice improvement.

Rice production in Ethiopia started few decades ago and now the country is proved to have reasonable potential to grow different rice types for lowland rain fed, upland and irrigated ecosystems. According to Shahi (1994), in Ethiopia a single rain fed upland crop could be grown in the altitudinal range of 1000 - 2000m. Around 250,000 hectare in the near future and about 1 million hectare in the distant future is expected to come under cultivation.

Institute of Biodiversity Conservation of Ethiopia has collection of rice species, among which some of them are wild rice species collected from both Fogera Plain, south Gonder, and Gambella region around Baro River but these collected species are not yet characterized.

Intensive collection and molecular marker based studies on wild rice species have been conducted in most of east, southern and other African countries except in Ethiopia (Kiambi *et al*., 2005; Brondani *et al.*, 2003; Ren *et al.*, 2003) although some indication shows the presence of Ethiopian wild rice species collection at IRRI (Ren *et al*, 2003) from Gambella and Gonder but the place where samples were collected and how samples were used in the study is ambiguous.

We can use the study of genetic variation to examine differences between members of the same species. On the other hand, we can compare the genetic composition of member of different species even over a wider taxonomic range (Dale and Schantz, 2002). Study about genetic variation is one of the most important methods for conservation of genetic resources. Different molecular markers can be used for direct measurements of genetic variation. Ultimately, comparison of complete genome sequences is the only way of detecting all the possible types of variation that may occur. However, we need quicker methods which are targeted at certain types of changes (Dale and Schantz, 2002).

Molecular marker techniques, like RAPD has been successfully employed to determine genetic diversity in many species including rice (Yu and Nguyen 1994; Ge *et al*., 1999). Qian *et al.* (2001) used RAPD marker to study genetic variation within and among populations of a wild rice *Oryza granulata* Nees et Arnex.Watt .The RAPD technique has several advantages such as a relatively unbiased portion of the genome sampled, simplicity of use, lower cost, and the use of a small amount of plant material (Fritsch and Rieseberg 1996). However, RAPD has several limitations including dominance, uncertain locus homology, and sensitivity to the reaction conditions, low reproducibility.

In order to solve some of these problems, a new technique, inter-simple sequence repeat amplification(ISSR), which involves PCR amplification of DNA using a single primer composed of a microsatellite sequence anchored at the 3’ or 5’ end by 2-4 arbitrary often degenerate, nucleotides, could be used to assess genetic diversity (Qian *et al.*, 2001).

ISSR has been successfully employed to assess genetic diversity within and between populations in several plant species. McRoberts *et al.* (2005), for instance, applied ISSR technique successfully in assessing genetic diversity of *Phalaris minor*. It was also found that double priming ISSR is an easy and informative genetic marker system in cotton for revealing both inter and intra specific variations (Liu and Wendell 2001). ISSR was also found better than RAPD to detect genetic diversity among the barley accessions since it amplifies the flanking sequence of microsatellite with longer SSR primer (Hou *et al.*, 2005).

As a dominant marker, ISSR targets simple sequence repeats (microsatellites) that are abundant through out the eukaryotic genome and that evolve rapidly. As a consequence, ISSR amplification reveals a much larger number of polymorphic fragments per primer than does RAPD. The technique also does not require prior knowledge of DNA sequence for primer design, and has advantages similar to those of RAPDs (Kantety *et al*., 1995).

Qian et al. (2001) concluded that, the two dominant DNA markers, RAPDs and ISSRs are effective and promising marker systems for detecting genetic variation. Furthermore, ISSR is superior to RAPD in terms of the polymorphic bands detected per primer and the reproducibility involved. The general objective of this study was therefore to analyze the genetic polymorphism and population genetic structure of wild populations of rice in Ethiopia and their relationship with cultivated rice species utilizing ISSR.

2. Literature Review

2.1. Origin and diversity of rice

Archaeological and historical evidence points to the foothills of the Himalayas in the North and hills in the North-east of India to the mountain ranges of South-east Asia and South-west China as the primary centre of origin of *Oryza sativa*, and the delta of River Niger in Africa for that of *O. glaberrima*, the African rice (Randhawa *et al.*,2006**)**..

O. perennis is a common ancestor for both *O. sativa* and *O. glaberrima*. The wild progenitors of *O. sativa* are the Asian common wild rice (*O. rufipogon*) which shows wide variation ranging in their habit from perennial to annuals. *O. sativa* is considered to have been domesticated between 9000 and 7000 BC. Schematic representation of evolutionary pathways of the Asian and African cultivated rice's is shown in Figure 3.1. The wild progenitor of the African cultivar *O. glaberrima* is *O. longistaminata* Chev.et Roehr endemic to West Africa. The primary centre of diversity for *O. glaberrima* is the swampy basin of Upper Niger (Randhawa *et al*., 2006).

Oryza is a modest sized genus consisting of 20 well recognized wild species and two advanced cultigens, *O. sativa* which is grown world wide and *O. glaberrima* grown in parts of West Africa (Chang, 2002) but more species are reported by (Purseglove, 1972). Wild *Oryza* species are distributed throughout the tropics. They can be grouped into four complexes of closely related species among which two species, the tetraploid *O. schlechteri* Pilger. and the diploid *O. brachyantha* Chev.et Roehr. seem to be different from the others (Maclean *et al*, 2002). These species and their chromosome numbers, genome group and geographic distribution are summarized in (Table3.1). Most of the species are diploid, having 12 pairs of chromosomes and seven species are tetraploids. Two species have both diploid and tetraploids sets of chromosomes.

According to Maclean *et al* (2002) species of *O. ridleyi* Hook. complex inhabit lowland swamp forests and species of the *O. meyeriana* (Nees et Mor.ex`Steud.) Baill.complex are found in upland hillside forests. The *O. officinalis* Wall ex Watt. complex consists of diploid and tetraploid species found throughout the tropics. All the species in this complex are perennial; some are rhizomatous and others form runners. They also differ in the habitats where they are found. Some occur in full sun, others in partial shade. Variation exists within these species as shown by the responses of different populations to pests and diseases. The *O. sativa* complex consists of the wild and weedy relatives of the two rice cultigens as well as the cultigens themselves. The wild relatives of *O. glaberrima* in Africa consist of the perennial rhizomatous species *O. longistaminata*, which grows throughout Sub-Saharan Africa, and Madagascar, and the annual *O. barthii* A. Chev., which extends from West Africa to East and Southern Central Africa. The annual and weedy relatives of *O. glaberrima* are found primarily in West Africa.

2.2. Taxonomy and botanical description

The genus *Oryza* belongs to the tribe Oryzeae of subfamily Oryzoideae in the family Poaceae (Gramineae) (Wang *et al.*1992). According to Purseglove (1972), there are 25 species of the genus *Oryza* in the tropical and sub tropical regions of Africa, Asia, Australia and South America, with the largest number in Africa, where there are 9 endemic species, and which is usually considered the center of origin of the genus. Diploid (2n=24) and tetraploid (2n=48) species are found, but the former are more numerous.

Rice plant vary in height from 40 to 70cm.The root system is fibrous with one seminal root, but adventitious roots can arise from nodes above the basal nodes. The rice culm is hollow with 10 to 20 internodes. An open leaf sheath arises from each node, and at the juncture of the leaf blade along ligule arises. Leaf blades are long and narrow with a characteristic long, hairy auricle. The rice leaf can be distinguished from those of other grasses by the presence of both ligule and auricle. Grain is produced on a loose panicle carried on the uppermost node of the culm that is most commonly completely free from the sheath. Seed is carried in 75 to 150 spike lets per panicle, each spikelet usually with a single floret. Florets contain six stamens, two feathery stigmas in two styles and two lodiculae. Panicles are 10 to 25 cm long. Rice is a self pollinated crop. Grain variations fit in to four categories: slender, long, medium and short (Stoskopf, 1985)

Table 2.1. Species of *Oryza*, chromosome numbers, genome group/symbol and geographic distribution (Source: Randhawa *et al*. 2006).

Complex/Species	**Chr. no.**	**Genome group**	**Distribution**
O.schlechteri Pilger	48	Tetraploid	Papua New Guinea
O.brachyantha Chev.et Roehr	24	FF	Africa
***O.ridleyi* complex**			
O.longiglumis Jansen	48	Tetraploid	Papua New Guinea
O.ridleyiI Hook	48	Tetraploid	Southeast Asia
***O.meyeriana* complex**			
O.granulata Nees et Arn ex watt	24	Diploid	South and South East Asia
O.meyeriana (Zoll.et Mor .ex Steud.)Baill	24	Diploid	South East Asia
***O.officinalis* complex**			
O.officinalis Wall ex watt	24	CC	Tropical Asia to Papua New Guinea
O.eichingeri Peter	24,48	CC	East and West Africa
O.rhizomatis D.A.Vaughan		CC	Sri Lanka
O.minuta J.S.Presl ex C.B.Presl	48	BBCC	Philippines, Papua New Guinea
O.punctata Kotschrex.Steud.	48,24	BB,BBCC	Africa
O.latifolia Desv.	48	CCDD	Central and South America
O.alta Swallen	48	CCDD	Central and South America
O.grandiglumis(Doell.) prod.	48	CCDD	South America
O.australiensis Domin	24	EE	Australia
***O.sativa* complex**			
O.glaberrima(cultigen) Steud.	24	AA	West Africa
O.barthii A.Chev.	24	AA	Africa
O.longistaminata Chev.et Roehr.	24	AA	Africa
O.sativa(cultigen) L.	24	AA	World wide
O.nivara	24	AA	Tropical Asia
O.rufipogon sensu lacto.	24	AA	Tropical Asia
O.meridionalis Ng	24	AA	Tropical Australia
O.glumaepatula Steud.	24	AA	South America

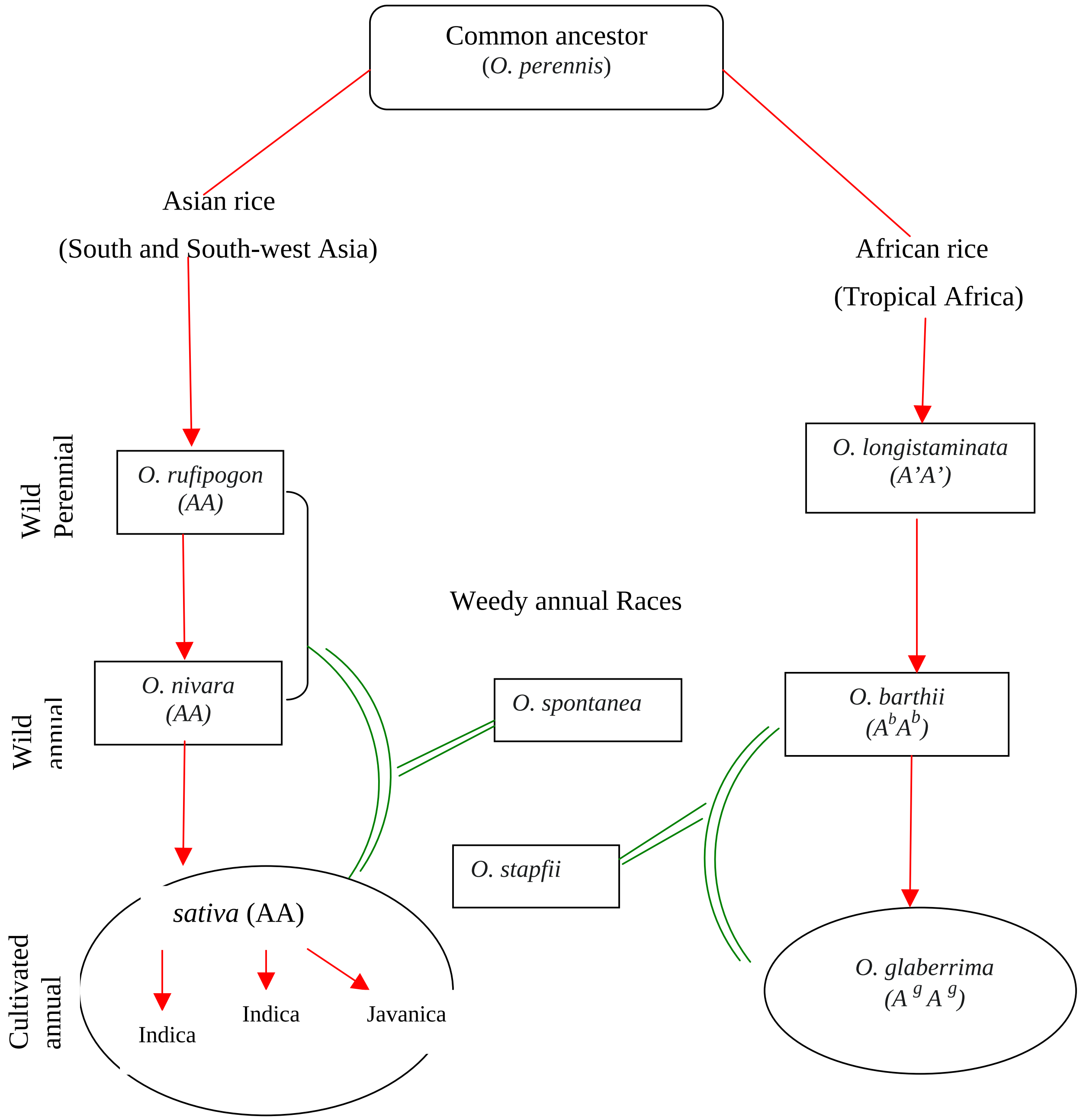


Figure 2.1. Schematic representation of the evolutionary pathways of Asian and African cultivated rice .Arrows with solid line indicates direct descent. Double lines indicate introgressive hybridization (Source: Randhawa *et al*., 2006**)**

2.3. Rice production systems

2.3. 1. Rice Husbandry and suitable agro ecology

Rice is grown under such widely differing conditions that it is difficult to define the climate that is most suitable for its development. One of the main reasons for this wide range of climatic conditions is the great diversity of rice cultivars. Except for Antarctica, every continent on the planet produces rice. It is grown from the equator to latitude 53^0 N (in china) and 40^0 S and from sea level to 3,000 m in the Himalayas. The chief limiting factor to its growth is not climate but the water supply (Onwueme *et al.*, 1991). Rice is grown on a variety of soils under varying climatic and hydrological conditions ranging from waterlogged and poorly drained to well-drained situations. Based on water regimes, rice can be classified as irrigated and rain fed rice, the rain fed ecosystem may be broadly categorized into upland and lowland ecologies. In the rain fed upland rice, there is no standing water in the field after few hours of cessation of rain. The lowland rice ecology depending on the water regimes may be further categorized into three sub ecologies:- shallow lowland rice: water depth below 50 cm,. semi-deepwater rice: - water depth between 50 to100 cm and deepwater rice:- water depth more than 100 cm in the field (Randhawa *et al*.,2006).

2.3. 2. Cropping patterns

In the rain fed upland and other moisture limiting/excess ecologies aimed at risk distribution and income enhancement intercropping of rice with appropriate companion crops help farmers to improve the productivity. According to Randhawa *et al*. (2006), some of the rice-based cropping patterns being followed in different rice ecologies includes:- **Rice-rice**:- this cropping pattern is practiced in the traditional rice growing areas having high rainfall and assured irrigation, **Rice-cereals** (other than rice):- this cropping pattern is being followed in areas where water is not adequate for taking the third rice crop, **Rice-pulses/oilseeds**:- in the areas where, there is water scarcity, **Rice-wheat-pulses**:- this cropping pattern is being followed in the alluvial soil and **Rice-fish farming system**:- Fields with sufficient water retaining capacity for a long period and areas free from heavy flooding are suitable for rice-fish farming system.

2.4. Cultivation and distribution of rice in Ethiopia

Cultivated rice (*Oryza sativa* L.) is only grown on a small scale as minor crop in Ethiopia (Phillips, 1995). However, recently several potential areas are identified for rainfed and irrigated rice types. According to CSA (2003) report, more than 8,326.50 hectare of land was under rice cultivation in 2003 with the yield of 154,120.39 quintal owned by 30,391 holders in Ethiopia (Table 3.2)

Table 2.2. Estimate of number of holders, geographic area, production and yield of rice in 2003 at country level (Source: CSA,2003)

Geographic area	Number of holders	Area (ha)	Production (Qt)	Yield (Qt/ha)
Amhara	23,155	6,599.88	131,929.98	19.99
Oromia	3,860	585.23	9,512.02	16.25
Benishangul Gumuz	2,747	1,102.82	12,293.79	11.15
SNNPR	301	*		
Gambella	329	38.57	384.60	9.97
Overall	**30,391**	**8,326.50**	**154,120.39**	

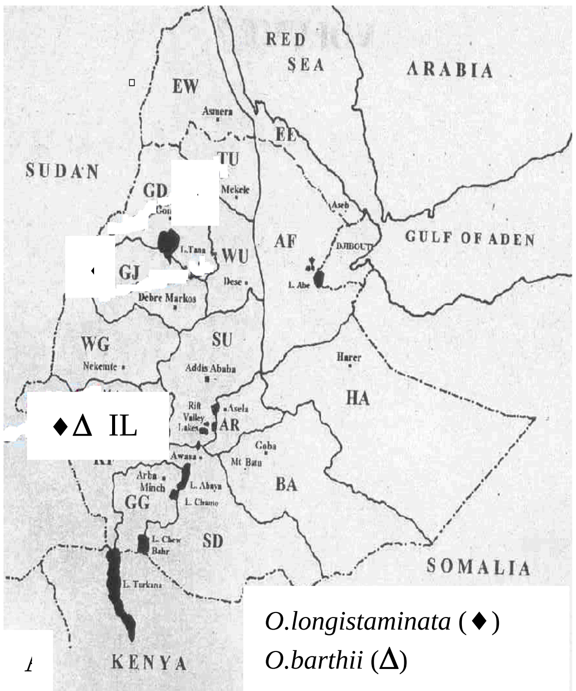
*-no data

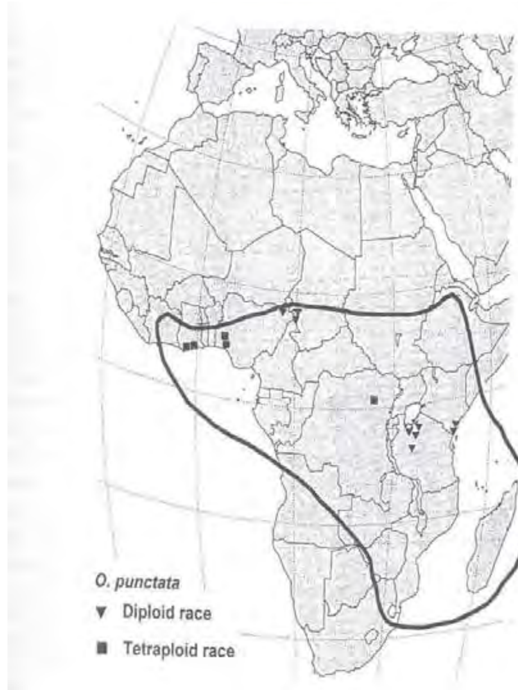
Recently more area coverage is expected because in areas like Afar and Somali, rice is a staple food,irrigated rice cultivation has been already started and rice research and promotion in all over the country is underway.

In Ethiopia there are also wild rice species in different parts of the country. According to Phillips (1995), two wild rice species were identified: *Oryza barthii* in Illubabor only and *Oryza longistaminata* in Gonder (GD), Gojam (GJ) and Illubabor (IL) (Figure 3.2a). Vaughan and Morishima (2002) also indicated that in addition to these two species a third species of wild rice, *O.punctata*, a species with two cytological forms (diploid and tetraploid) is found in Ethiopia (Figure 3.2b and Figure 3.2c).

Moreover, genus *Leersia* which belong to the same Tribe as genus *Oryza* and as very close relationship with rice is also reported to be found in Ethiopia (Phillips, 1995).

Genus *Leersia* is spread allover the country as compared to very restricted distribution of wild rice.





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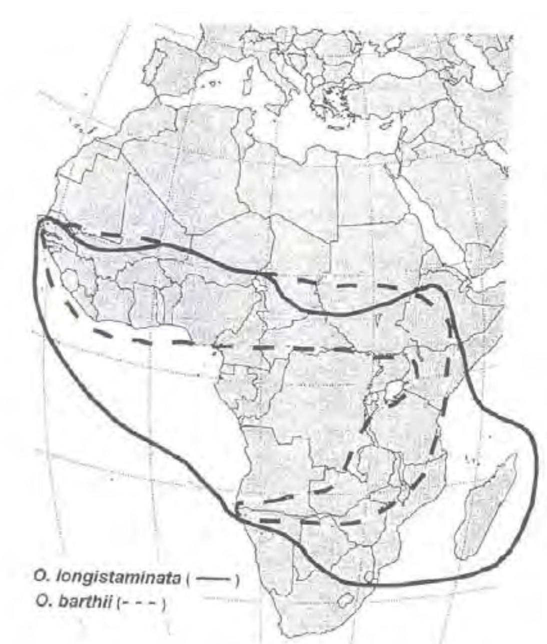

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Figure 2.2: Distribution of wild rice species in Ethiopia and Africa

A. Distribution of *O. barthii* A.Chev. and *O. longistaminata* Chev.et Roehr in Ethiopia,

B. Distribution of *O.punctata* Kotschy ex Steud in Africa and

C. Distribution of *O.barthii* and *O.longistaminata* in Africa.

Source: (Phillips, 1995; Chang, 2002)

2.5. Loss of genetic diversity and conservation of rice genetic resources

2.5.1. Loss of genetic diversity

Genetic diversity is required for populations to evolve and cope up with environmental change, new disease and pest epidemics. Loss of genetic diversity is often associated with reduced reproductive fitness (Frankham *et al*, 2002). As the green revolution in rice was centered largely on the sd-1 gene and the cytoplasm of the cultivar cina (China), the genetic base of improved O. *sativa* cultivars allover the world became greatly narrowed. Meanwhile, numerous traditional types were displaced and lost from farmers' fields. Concurrently, disturbance or destruction of many natural habitats has reduced the populations of wild relatives. The continuing trend toward erosion of genetic diversity is worrisome in the face of pest epidemics under intensive cultivation and global climate change (Chang, 2002).

In many areas, high-yielding modern varieties were adopted by farmers and the cultivation of the landrace varieties declined. The wild species are threatened with extinction through changes in land use, extension of agriculture into marginal areas and deforestation (Jackson, 1997).The center of origin of rice species are characterized by topological heterogeneity and are considered to be the centers of rice diversity. The diversity in these centers is being lost rapidly with many rice growers shifting to modern cultivars (Randhawa *et al*.,2006**).**

The wild rice genetic diversity is currently also eroded because of various reasons like for example grazing and agricultural activities in most of the countries. For instance, in countries such as Malawi and Uganda, increased subsistence rice production is continually leading to destruction of habitats where wild rice grows (Kiambi *et al*, 2005). In addition, the wild species are threatened by their eradication from farm fields because it is considered as weeds.

2.5.2. Conservation of rice genetic resources.

According to Frankham *et al*. (2002) we conserve biodiversity because of the economic value of bioresources, ecosystem services, aesthetics and rights of living organisms to exist. The problems of genetic erosion are severe; however, international efforts to conserve rice genetic resources, in which IRRI has taken a leading role, have led to the establishment of several gene banks in Asia. These joint efforts between national, regional and international organizations ensure the long term preservation of the diversity of the rice gene pool (Jackson, 1997).

For many plant species, *ex situ* conservation of seeds is safe and cost effective, provided proper attention is paid to seed drying and storage conditions. Fortunately, rice seeds exhibit orthodox storage behavior and can be dried to a low moisture content of ca. 6% and stored at -20^{0}C, retaining their viability for decades, if not longer (Jackson,1997).

Molecular approaches has also great role in germplasm conservation and it adds another dimension to the study of genetic diversity in germplasm collections. In the international rice gene bank (IRG), a range of biochemical and molecular approaches is used to study the diversity of conserved germplasm. Such approach does facilitate the use of rice varieties when morphological identification is ambiguous. Molecular markers such as RAPD do offer an opportunity to identify duplicate accessions, the use of other molecular markers may also assist in the development of a core collection for rice- a sub set of the whole collection that represents the complete diversity of the genus *Oryza* (Jackson, 1997).

2.6. Marker systems and their applications in genetic diversity analysis

2.6.1. Morphological markers

Morphological markers are a marker system based on phenotypic appearance. It is the earliest genetic markers used for assessment of variation and still has great importance. Moreover, morphological characters are simple to score and inexpensive too.

The main disadvantages of this approach are the influence of the environment, extra time and resources needed for evaluation in the field and greenhouse. Moreover, some morphological traits are observed to be very plastic in nature which could be easily affected by environmental change and affect the exact relationships of plant species and/or populations (Vithanage *et al.*, 1995).

2.6.2. Biochemical markers

Biochemical markers are markers based on protein polymorphisms through electrophoretic separation of protein molecules. A tissue extract is prepared and electrophoresed on a non denaturing starch or polyacrylamide gel. The proteins of this extract are separated by their net charge and size. After electrophoresis, the position of a particular enzyme in the gel is detected by adding a colorless substrate that is converted in to a dye under appropriate reaction conditions. Depending on the number of loci, their state of homo-or heterozygosity, and the enzyme configuration (i.e., the number of separable units), from one to several bands are visualized. The positions of these bands can be polymorphic and can be considered as informative loci (Weising *et al*., 2005).

The main advantages of protein markers are their codominant inheritance and the technical simplicity and low cost of the assay. Disadvantages include the restricted number of suitable allozyme loci in the genome, the requirement of fresh tissue, and the sometimes limited variation (Weising *et al*., 2005).

2.6.3. Molecular markers

Molecular markers are based on naturally occurring polymorphisms in DNA sequences (i.e.: base pair deletions, substitutions, additions or patterns) (Gupta, 1999). Molecular markers are superior to both morphological and biochemical markers because they are relatively simple to detect, abundant throughout the genome even in a highly inbred cultivars, completely independent of environmental conditions and can be detected at virtually any stage of plant development.

According to Vithanage *et al.* (1995) molecular markers are "land marks" which can be identified on the genome and, therefore, offer the best possible means of identifying individuals from biological samples. Molecular markers can be applied in the identification of cultivars and clones, genetic mapping, marker assisted selection (MAS), population genetics, molecular systematics and etc. (Weising *et al*., 2005).

In recent years different marker systems such as Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Polymorphic DNAs (RAPDs), Sequence Tagged Sites (STS), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs) or micro-satellites, Single Nucleotide Polymorphisms (SNPs) and others have been developed and applied to a range of crops including cereals (FAO, 2003). The relative advantages and disadvantages of these techniques are summarized below.

Table 2.3: Comparison of the most commonly used molecular marker systems in cereals (FAO, 2003).

Feature	**RFLPs**	**RAPDs**	**AFLPs**	**Microsatellites (SSRs)**	**SNPs**
DNA required (µg)	10	0.02	0.5-1.0	0.05	0.05
DNA quality	high	High	Moderate	Moderate	high
PCR based	no	Yes	Yes	Yes	yes
Number of polymorphic loci analyzed.	1.0-3.0	1.5-50	20-100	1.0-3.0	1.0
Ease of use	Not easy	Easy	Easy	Easy	easy
Amenable to automation	low	moderate	Moderate	High	high
Reproducibility	high	unreliable	High	High	high
Development cost	low	Low	Moderate	High	high
Cost per analysis	high	Low	Moderate	Low	low
Dominance/codominance	codominant	dominant	Dominant	Codominant	codominant

2.6.3. 1. Restriction Fragment Length Polymorphism (RFLP)

In RFLP analysis, DNA is extracted from organism of interest, and digested with one or more restriction enzymes. The resulting fragments are separated according to size by gel electrophoresis. The gel is southern-blotted on to a membrane, and one or more specific fragments are visualized by blot hybridization with a labeled probe.

The main advantages of RFLP markers are their codominance and high reproducibility. However, the drawbacks as compared with PCR- based techniques is that the requirement of relatively pure and intact DNA (Weising *et al.*, 2005), technically laborious and expensive.

2.6.3.2. Random Amplified polymorphic DNA (RAPDs)

Random amplified polymorphic DNA (RAPD) markers were first described in 1990 (FAO, 2003). RAPD was the first PCR based molecular marker technique developed and it is by far the simplest. Short PCR primers (approximately 10 bases) are randomly and arbitrarily selected to amplify random DNA segments throughout the genome. The resulting amplification product is generated at the region flanking a part of the 10 bp priming sites in the appropriate orientation. RAPD often shows a dominant relationship due to primer being unable to bind (show 3:1 ratio, unable to distinguish between homozyogotes and heterozygotes) (Yin *et al*, 2001).

Advantage of RAPD is its technical simplicity, paired with the independence of any prior DNA sequence information. However, its dominant nature and less reproducibility are its main disadvantage (Weising *et al*., 2005).

2.6.3.3. Amplified Fragment Length Polymorphism (AFLPs)

In the mid 1990's, another PCR-based method of molecular markers was described, giving rise to amplified fragment length polymorphism (AFLP) markers. With this technique, the DNA treated with restriction enzymes is amplified with PCR. It allows selective amplification of restriction fragments giving rise to large numbers of useful markers which can be located on the genome relatively quickly and reliably (FAO, 2003).

AFLP can be used to distinguish closely related individuals and can also map genes. Like that for RFLP, high quality DNA is also required. AFLP is extremely sensitive technique and the added use of fluorescent primers for automated fragment analysis systems, and sophisticated software packages to analyze the biallelic data, makes the AFLP well suited for high throughput analysis (Farooq *et al*, 2002).

The major advantage of the AFLP technique is generation of a large number of polymorphism. The fact that no sequence information is required and that the PCR technique is fast with a high multiplex ratio makes the AFLP very attractive choice (Rafalski *et al.*, 1996).

2.6.3.4. Microsatellites

These are simple DNA sequences (e.g. AC), usually 2 or 3 bases long, repeated a variable number of times in tandem. They are easy to detect with PCR and a typical microsatellite marker has more variants than those from other marker systems. Initial identification and screening of microsatellite loci are time-consuming (FAO, 2003).

Microsatellites are ideal genetic markers for detecting differences between and within species. It consists of tandomly repeated 1-7 base pair units, distributed widely throughout the genome. These are heritable, useful to monitor gene flow, excellent for parentage determination and ideally suitable for analysis via multiplexing with highly reproducible profiles (Farooq *et al*, 2002). The variation in the number of tandomly repeated units results in highly polymorphic banding patterns. Profiles are generated by PCR amplification of unique loci using discriminatory primers sets.

Microsatellites may even be used across species and genus boundaries. Microsatellites are not limited to the nuclear genome. They occur in chloroplast as well as in mitochondrial genome (Soranzo *et al*., 1999). Nuclear microsatellites are of bi-parental inheritance and possess few loci with many alleles per locus. They are codominant with exception of null alleles at some loci, can detect large variation within population confer stability to the genome (Farooq *et al*, 2002).

Chloroplast microsatellites on the other hand are of uni-parental inheritance with single locus (many alleles per locus), are non-coding and detect low variation within population and large variation between populations. Their genome does not recombine due to its paternal inheritance in conifers (Sperisen *et al*., 1998) and maternal inheritance in angiosperms (Dumolin *et al*., 1995). For this reason, chloroplast microsatellites (cp) short tandem repeats (STR) variants accumulated in a uni-parental chloroplast lineage can provide information about the history of tree population. Mitochondrial micro-satellites are also of uni-parental (maternal), single locus inheritance with many alleles per locus. They are also non-coding and can detect low variation within population but large variation between regions (Farooq *et al*., 2002).

Chloroplast microsatellite variants are supposed to be generated in a stepwise manner by addition or deletion of a single repeat unit. Under such a stepwise mutation model microsatellite variants with small repeat length differences are more closely related than alleles with larger length differences (Jarne *et al.*, 1996).

2.6.3.5. Single Nucleotide Polymorphisms (SNPs)

SNPs are single base pair positions in the genomes of two or more, at which different sequence alternatives (alleles) exist in populations (Weising *et al.,* 2005). In recent years, single nucleotide polymorphisms (SNPs), i.e. single base changes in DNA sequence, have become an increasingly important class of molecular marker. The potential number of SNP markers is very high, meaning that it should be possible to find them in all parts of the genome, and micro-array procedures have been developed for automatically scoring hundreds of SNP loci simultaneously at a low cost per sample (FAO, 2003).

2.7. Inter Simple Sequence Repeats (ISSR) markers in genetic diversity analysis

The inter simple sequence repeats (ISSR) are a new kind of molecular marker and glorified RAPD involving PCR amplification of DNA by a single primer (16-18) bp long composed of repeated sequence anchored or nonanchored at the 3' or 5' end by 2-4 arbitrary nucleotides (Zietkiewicz *et al*., 1994). They are easy to handle, highly informative and repeatable.

ISSRs are now being applied for cultivar identification, assessment of genetic diversity in various plant species (Hou *et al.*, 2005; Pomper *et al.*, 2003) and in determining genetic diversity and phylogenetic relationships in *Oryza* (Joshi *et al.,*2000).

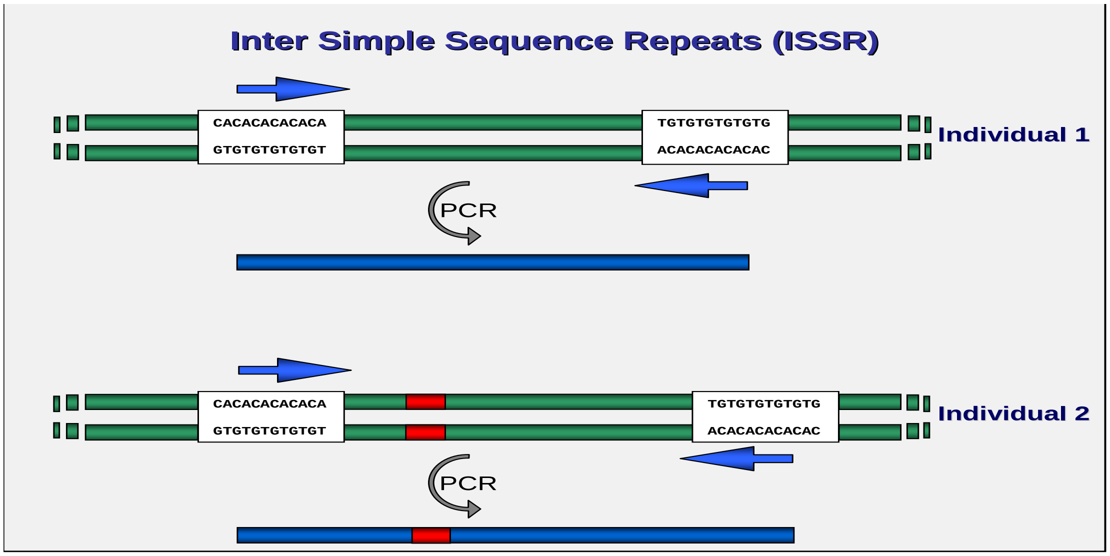


Figure 2.3. Principles of the amplification of DNA with a single oligo-nucleotide primer in ISSR marker system. DNA segments delimited by the inverted simple sequence repeats (SSR) (Individual 1 and 2) are amplified with a single ISSR primer (green). ISSR Variation that may result from insertion or deletion (Red mark) in different individuals produces PCR fragments of deferent sizes (blue) of the segment (Tesfaye *et al*., 2005)

3. Objectives:

3.1. General objective:

- To analyze the genetic polymorphism and population genetic structure of wild populations of rice in Ethiopia and their relationship with cultivated rice species utilizing ISSR.

3.2. Specific objectives:

- To determine genetic distance and identity within rice species and the cultivars studied based on ISSR marker.
- To determine genetic distance and identity between rice species based on ISSR marker.

4. Materials and Methods

4.1. Plant Materials

A total of 60 samples representing six populations were collected from various areas. Sampling was done randomly with approximately 10 m distance from each other. Two cultivated rice species, representing two populations (one *O. sativa*, one NERICA; NERICA-3 and two wild rice populations; wild Gonder-1 (*O. barthii*) and Wild Gonder-2 (*O. longistaminata*) were collected from south Gonder, North west Ethiopia, one wild rice population (*O. longistaminata*) collected from Baro river side, Gambella (west Ethiopia) (Figure 4.1) and *Oryza glaberrima*, African rice species was provided by Rice Regional Office, $SG_{2000,}$ in Ethiopia which was originally brought from West Africa. The lea samples of wild rice populations, *O. sativa* and NERICA were collected and dried in silica gel for DNA extraction. However, the seed of *O. glaberrima* were grown in the greenhouse and the leave samples were further collected and dried in silica gel for extraction of genomic DNA.

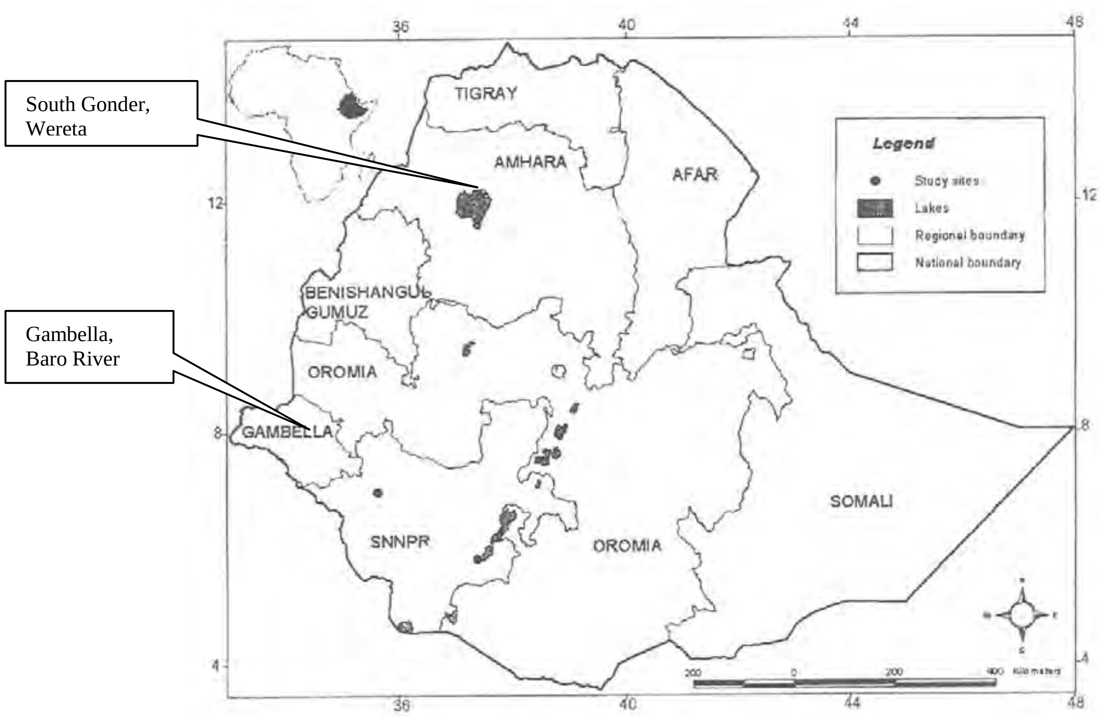


Figure 4.1: Map of Ethiopia showing sites from where samples of rice were collected, which are also among the potential areas for rice production.

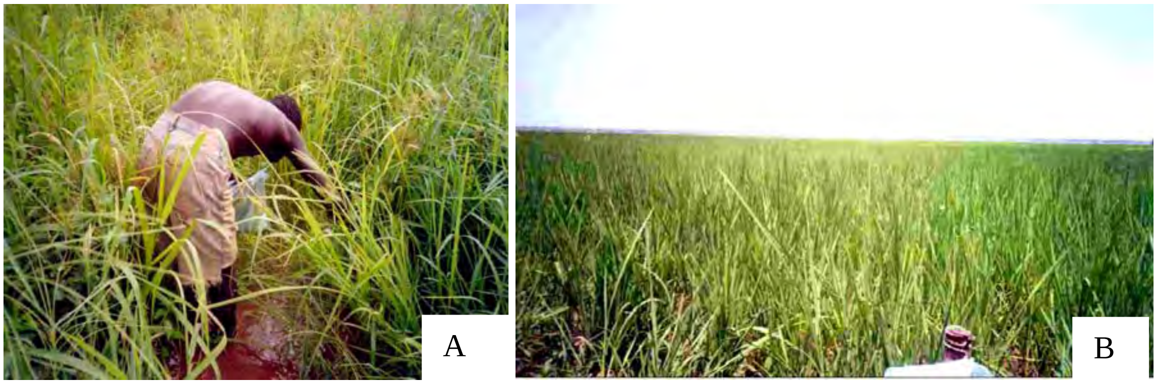


Figure 4.2: Wild rice at Gambella, Baro river side (A), and cultivated rice field at Wereta, North of Lake Tana, South Gonder (B).

4.2. DNA extraction

Genomic DNA extraction was done based on the method used by (Borsch *et al*., 2003) which involves a modified CTAB method employing triple extractions to yield optimal amounts of DNA.

4.3. Test gel and electrophoresis

An agarose gel (100ml, 1xTBE and 0.98g agarose) was prepared and 2 µl each genomic DNA samples with 6µl loading dye was loaded on to the gel and electrophoresed at constant voltage of 80V for 45 minutes. The gel was stained for 30 min with 50 µl ethidium bromide (10mg/ml) after well mixed with 450 ml distilled water then de-stained for 30 minutes with distilled water. Gel picture was taken under UV transilluminator by BiodocAnalyse 2.0 with digital canon camera. From the three extractions following the protocol given by (Borsch *et al*, 2003) those with high band intensity and less smear were selected for PCR, and this was commonly the case for the second extractions.

4.4. Primer selection and optimization

A total of eight ISSR primers obtained from the University of British Colombia (primer kit UBC 900) and primers used by (Sujatha *et al.* 2004) were used for the initial testing of variability and reproducibility test. One individual selected from each population to screen the primers with 1:5 dilutions and finally six primers were selected and these include 4 di-nucleotides and 2 tetra- nucleotides repeat motives (Table 4.1) based on polymorphism and reproducibility.

Table 4.1:- List of primers, annealing temperature and sequence. All the primers are high performance liquid chromatography purified and designated as 'H'

Primers	*Annealing temperature*	*Sequence*
810- H	45^0C	**5'-GAG AGA GAG AGA GAG AT-3'**
818- H	48^0C	**5'-CAC ACA CAC ACA CAC AG-3'**
824- H	48^0C	**5'-TCT CTC TCT CTC TCT CG -3'**
834- H	45^0C	**5'-AGA GAG AGA GAG AGA GYT -3'**
854- H	48^0C	**5'-TCT CTC TCT CTC TCT CRG -3'**
872- H*	35^0C	**5'-GAT AGA TAG ATA GAT A -3'**
873- H*	45^0C	**5'-GAC AGA CAG ACA GAC A -3'**
878- H*	**45^0C**	**5'-GGA TGG ATG GAT GGA T -3'**

* -Tetranucleotide primers and the rest are dinucleotides.

4.5. PCR and gel electrophoresis

The polymerase chain reaction was conducted in Biometra 2000 T3 Thermo cycler. PCR amplification was carried out in a 25 µl reaction mixture containing 1µl template DNA, 13.2µl H_20, 5.6µl dNTP (1.25mM), 2.6µl Taq buffer (10xThermopol reaction buffer), 2.0µl $Mgcl_2$ (2mM), 0.4µl primer (20pmol/µl) and 0.2µl Taq Polymerase (5u/µl). The amplification program was 4 minutes preheating and initial denaturation at 94^0C, then 39 x 15 seconds at 94^0C, 1 minute primer annealing at (45^0C/ 48^0C) based on primers used, 1.30 minutes extension at 72^0C. The final extension for 7 minutes at 72^0C followed. The PCR reactions were also stored at 4^0C until loading on gel for electrophoresis.

An agarose gel (1.67% agarose with 100 ml 1xTBE) prepared and 8µl amplification product of each sample with 2µl loading dye was loaded on gel. DNA seizer or DNA marker 500 bp was used to estimate molecular weight. The electrophorese were done for 2 hours at constant voltage of 100V. Gel picture was taken after staining with ethidium bromide (10mg/ml) which was mixed with 450 ml distilled water for 30 minutes and destained for 30 minutes.

4.6. Data scoring and Analysis

Each fragments that was amplified using ISSR primers, was treated as a unit character and scored as '0' for absence, '1' for presence and '?' for missing data. Based on recorded bands different software's were used for analysis. POPGENE version1.32 software (Yeh *et al.*, 1999) was used to calculate genetic diversity for each population as number of polymorphic loci and percent polymorphism. Analysis of molecular variance (AMOVA) (Excoffier *et al.*, 2006) was used to calculate variation among and within population using Areliquin version 3.01 (Excoffier *et al.*,2006).

Shannon–Weaver diversity index (H) was calculated as H= -∑pilog2pi where pi is the frequency of a given band for each population (Lewontin, 1972). Shannon's index of diversity was used to measure the total diversity (Hsp) as well as the mean intra – population (Hpop). The proportion of diversity between populations was then calculated as (Hsp-Hpop/Hsp).

NTSYS- pc version 2.02 (Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek *et al.*, 1999) software's were used to calculate Jaccard's similarity coefficient which is calculated with the formula:-

$$S_{ij} = \frac{a}{a+b+c}$$

Where,

'a ' is the total number of bands shared between individuals i and j,

'b' is the total number of bands present in individual i but not in individual j and

c is the total number of bands present in individual j but not in individual i.

The unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973) was used to analyze and compare the individual genotypes and generates phenogram using NTSYS- pc version 2.02 (Rohlf, 2000). The neighbor joining (NJ) method (Saitou and Nei1987; Studier and Keppler, 1988) was used to compare individual genotypes and evaluate patterns of genotype clustering using Free Tree 0.9.1.50 Software (Pavlicek *et al.*, 1999). The major difference between the two algorithms is that UPGMA assumes equal rates of evolution (molecular clock assumption) along all branches, whereas neighbor joining assume variations in the rate of change (Saitou and Nei1987; Studier and Keppler, 1988; Nei and Kumar 2000; Lan and Reeves 2002).

To further examine the patterns of variation among individual samples, a principal coordinated analysis (PCO) was performed based on Jaccard's coefficient (Jaccard, 1908). The calculation of Jaccard's coefficient was made with PAST soft ware version 1.18 (Hammer *et al.,* 2001). The first three axes were later used to plot with STATISTICA version 6.0 software (Hammer *et al.,* 2001; statistica soft, Inc.2001).

5. Results

5.1. Banding patterns and ISSR primers

Out of the eight primers tested initially, six of them gave relatively clear banding pattern and they were selected and used in this study (Table 5.1). The molecular weight of the bands amplified using 6 primers were in the range of 450 bp to 4kb (Figure5.1). A total of 93 bands were scored from 6 primers in which the least 12 bands were scored from 854_H and the highest were scored from 810_ H (19) in di-nucleotides. Of all primers higher number of bands(22) were scored from tetra-nucleotides (873_H).

Table 5.1:-Fingerprint patterns generated using eight ISSR primers; six of them are selected for this study.

Primers used	Repeat motif	Amplification pattern	Amplification of specific bands	Number of scorable bands
810- H	**$(GA)_8T$**	**Good**	**Good with smear**	19
818- H	**$(CA)_8G$**	**Smeared**	**-**	-
824- H	**$(TC)_8$ G**	**Good**	**Good**	16
834- H	**$(AG)_8$ YT**	**Good**	**Good with smear**	15
854- H	**$(TC)_8$ RG**	**Good**	**Good**	12
872- H	**$(GACA)_4$**	**Only some samples are amplified**	**-**	-
873- H	**$(GACA)_4$**	**Only one sample amplified (further optimization required)**	**Good for amplified one**	22
878- H	$(GGAT)_4$	Good but with smear	Good with smear	9

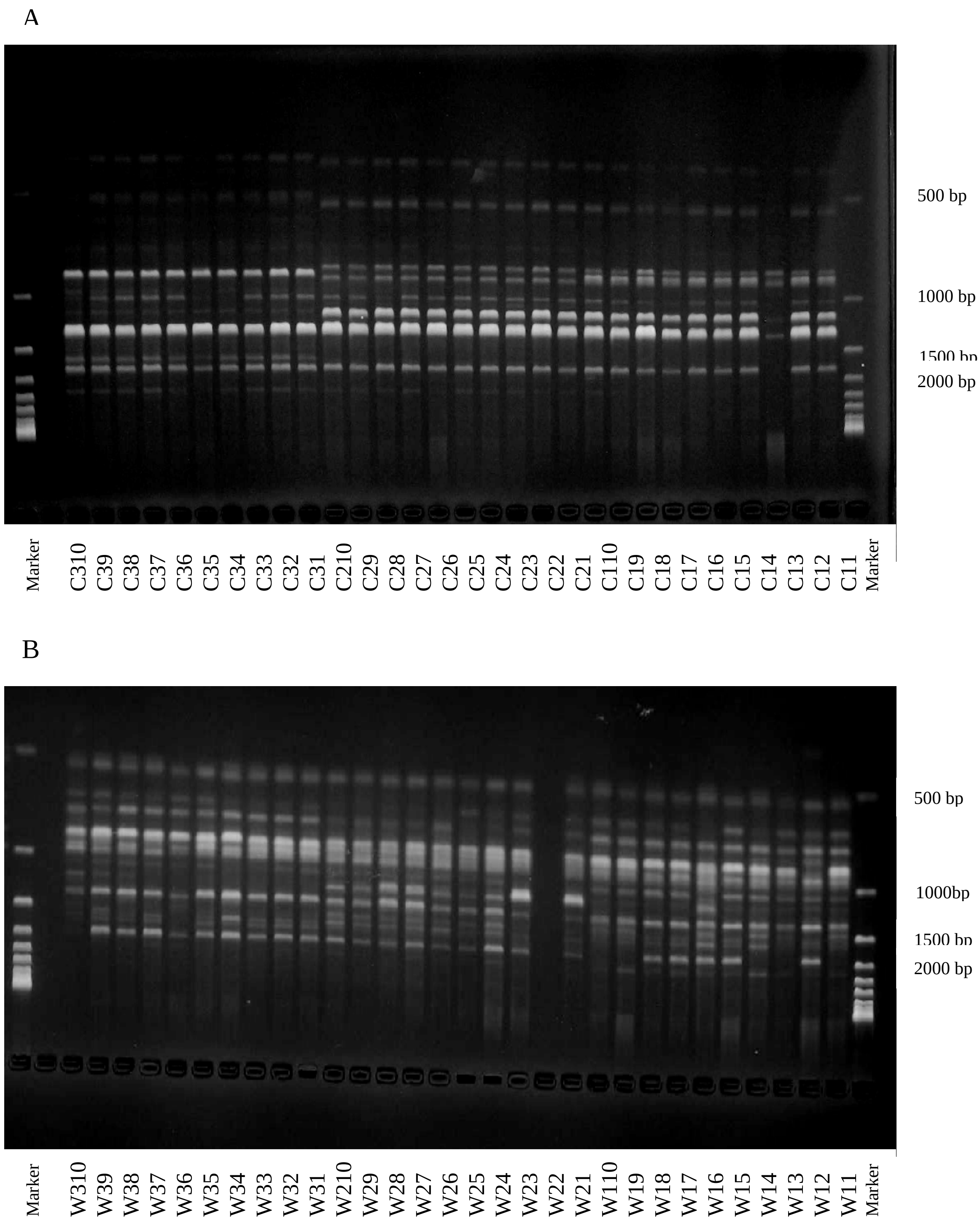


Figure5.1. ISSR fingerprint generated from 30 cultivated rice (A) and 30 wild rice populations (B) using primer 873-H

5.2. ISSR band specificity of rice populations

As shown in Table 5.2, locus 9 and 15 of primer 824 and 873 respectively, was found to be specific fragments for Wild Gonder -1 population, locus 3 of primer 810 and locus 1,3,5,9 of primer 854 was found specific for NERICA population and locus 9 and 22 of primer 873 was found to be specific for *O. glaberrima* rice population.

Table 5.2: List of specific bands for rice populations studied

Population	**Locus**
Wild Gonder-1 (*O. barthii*)	824-9, 873-15
NERICA- 3	810-3, 854-1, 854-3, 854-5, 854-9
O. glaberrima	873-9, 873-22
O. sativa	-
Wild Gonder-2 (O. longistaminata)	-
Wild Gambella (O. longistaminata)	-

5.3. Polymorphism

The number of polymorphic loci ranges from 9 for 878_H to 22 for 873_H and both primers are tetra repeat motif. For dinucleotide primers the polymorphic loci ranges from 12 for 854_H to 19 for 810_H. Likewise, 96.77% of the total loci scored were observed to be polymorphic. Three dinucleotide primers, namely 810- H, 824- H and 834- H showed 100% polymorphism. In contrast, 878- H showed the least polymorphism which was 88.89% (Table5.3). Among all the populations studied *O. glaberrima* and wild Gonder- 1 were found to have higher percentage polymorphism among cultivated and wild rice species, respectively. Generally, wild rice populations were observed to show higher percentage polymorphism as compared to cultivars.

5.4. Gene diversity

Among cultivated rice species considered in the present study, NERICA has higher gene diversity (0.12) than both *O. sativa* (0.11) and *O. glaberrima* (0.11). Wild Gonder- 1 has higher gene diversity among wild rice populations in the present study with gene diversity value of 0.18 (Table5.3). Generally wild rice populations were found to have higher gene diversity (0.14) than cultivated rice populations with over all gene diversity of 0.11.

Table 5.3:- Number of polymorphic loci, percent polymorphism and genetic diversity of both wild and cultivated rice with each and all primers.

Population	**With all primers**		
	NPL	**PP (%)**	**GD**
NERICA	25	26.9	0.12
O.sativa	26	28.0	0.11
O.glaberrima	28	30.1	0.11
Average (cultivated)		**28.3**	**0.11**
Wild Gnder_1 (*O.barthii*)	43	46.2	0.18
Wild Gonder_2 (*O.longistaminata*-1)	32	34.4	0.13
Wild Gambella (*O.longistaminata*-2)	32	34.4	0.12
Average (wild)		**38.3**	**0.14**
Over all	**90**	**96.77**	**0.34**
For individual primers			
810_H	19	100	0.40
824_H	16	100	0.34
834_H	15	100	0.35
854_H	12	91.67	0.22
873_H	22	95.45	0.30
878_H	9	88.89	0.35

NPL= number of polymorphic loci, PP (%) = percent polymorphism and GD= gene diversity for each population and over all populations.

5.5. Shannon's diversity index

The overall analysis with both di and tetra nucleotide primers indicated that the wild rice collected from Gonder (wild Gonder- 1) was found to be more diverse as compared to the other two, wild Gonder- 2 and wild Gambella. From cultivated rice species *O. glaberrima,* African rice, which was brought from West Africa, showed higher genetic diversity as compared to the other two cultivated rice populations with least diversity in *O. sativa* (0.20) as shown in (Table 5.4).

Generally, wild rice show higher genetic diversity than cultivated rice. The variation in genetic diversity between wild rice populations was found greater than that of cultivated. Higher genetic diversity were observed among populations (63%) of rice as compared to within which is 27%. The mean genetic diversity for populations and mean genetic variation for the entire data was higher with di-nucleotide primers as compared to tetra-nucleotide primers but the mean Shannon diversity level revealed by dinucleotide (0.28) was not significantly higher than the Shannon diversity level of tetranucleotide primer (0.23) with 95% confidence interval at $t(10)=1.67$, P-value=0.156.

Table 5.4. Shannon's diversity index within and among rice populations with di and tetra nucleotide primers.

Popn/spps	Shannon's diversity index (H)		Over all H
	Di- primers	Tetra– primers	
NERICA	0.257	0.122	0.212
O.sativa	0.243	0.120	0.202
O.glaberrima	0.262	0.178	0.234
wild Gonder_1 (*O.barthii*)	0.370	0.384	0.375
Wild Gonder_2 (*O.longistaminata*_1)	0.315	0.261	0.297
Wild Gambella (*O.longistaminata*_2)	0.249	0.312	0.270
Hpopn	**0.283**	**0.230**	**0.265**
Hsp	**0.799**	**0.734**	**0.777**
Hpopn/Hsp	**0.370**	**0.353**	**0.365**
1-Hpopn/Hsp	**0.630**	**0.615**	**0.625**

Hpopn =mean genetic variation for popn, Hsp=mean genetic variation for the entire data, Hpopn/Hsp =proportion of genetic variations within rice populations and 1-Hpopn/Hsp = proportion of genetic variations between rice populations.

5.6. AMOVA

Analysis of molecular variance revealed that higher percentage of variation is attributed to variation among populations within groups (49.4%) and followed by variation among groups and within populations with 26.4% and 24.2%, respectively (Table 5.5b). AMOVA also revealed that variation among populations within groups, variation among groups and within populations was found highly significant at (P=0.00). Similarly without grouping (Table 5.5a) high variation is attributed to among the six rice populations with high fixation index (0.73) with least variation within populations.

Table 5.5 A: Analysis of Molecular Variance (AMOVA) of rice populations in Ethiopia without grouping. B: Analysis of Molecular Variance (AMOVA) of cultivated and wild rice groups.

A

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation Indices	P
Among Populations	5	643.083	12.4007Va	72.90	0.73	0.00
Within Populations	54	248.900	4.60926Vb	27.10		0.00
Total	59	891.983	17.010			

B

Source of variation	d.f	Sum of squares	Variance components	Percentage of Variation	P
Among Groups	1	248.950	5.01389 Va	26.37	0.00
Among populations within groups	4	394.133	9.39241 Vb	49.39	0.00
Within Populations	54	248.900	4.60926 Vc	24.24	0.00
Total	59	891.983	19.01556		

5.7. Genetic Similarity

Based on Jaccard's coefficients of similarity indicated in (Table 5.6) and (Figure5.2) high similarity was observed between *O.sativa* and NERICA rice species (0.53) followed by wild rice from Gambella and wild Gonder-2(0.50), the least similarity was found between wild Gambella with *O.sativa* and NERICA having the value of 0.28 and 0.29 , respectively. *O.glaberrima*, an African rice population, in this study generally shows more genetic similarity to cultivars than wild rice populations as a result it clustered with cultivars.

Table 5.6:- Similarity matrix for Jaccard's coefficients for 6 rice populations based on the 93 bands obtained with 6 ISSR primers.

Popn	NERICA	*O. sativa*	*O. glaberrima*	WGonder-1 (*O.barthii*)	WGonder-2 (*O.lonistaminata*)	WGambella (*O.lonistaminata*)
NERICA	1.00					
O.sativa	0.53	1.00				
O.glaberrima	0.38	0.48	1.00			
WGonder-1	0.39	0.45	0.44	1.00		
WGonder-2	0.35	0.38	0.31	0.48	1.00	
WGambella	0.29	0.28	0.31	0.48	0.50	1.00

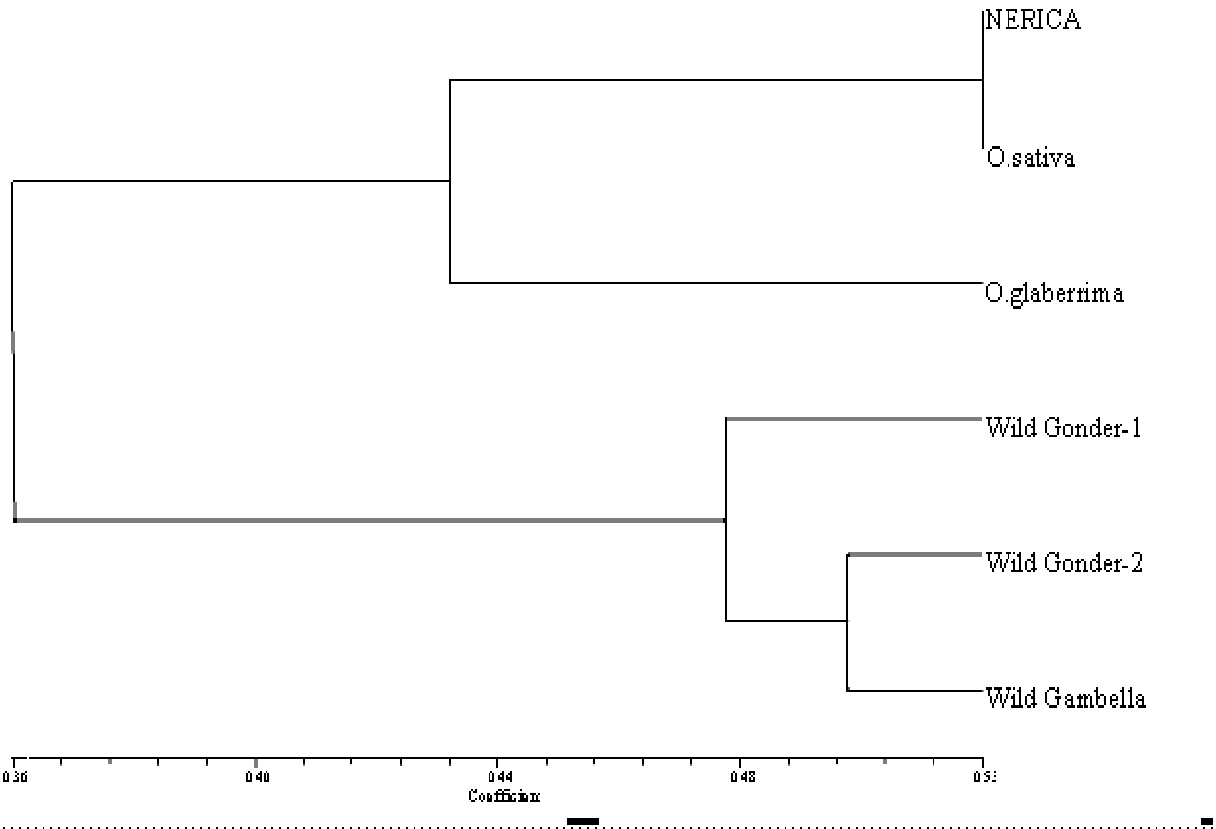


Figure5.2. UPGMA based dendrogram for 6 rice populations using 6 ISSR (4 di and two tetra nucleotide) primers.

5.8. Clustering Analysis

UPGMA and neighbor joining analysis was used to construct dendrogram for 60 individuals based on 93 bands obtained with 4 di- and 2 tetra-nucleotide primers, accordingly in both dendrogram the six populations (NERICA, *O. sativa*, *O. glaberrima*, wild Gonder_1, Wild Gonder- 2 and Wild Gambella) appeared to have their own cluster separately (Figure5.3 and 5.4). NERICA, a species of rice developed through inter-specific hybridization of Asian and African rice form cluster with Asian rice. From wild species, Wild Gonder- 2 found with the same group with wild Gambella. Generally cultivated and wild species clearly observed to have separated groups in both UPGMA and neighbor joining analysis. Both trees recovered almost the same tree topology with similar groupings, although few individuals appeared to escape from groups in case of neighbor-joining analysis.

African rice, *O. glaberrima*, which is expected to form cluster with wild rice populations based on evolutionary pathways is clustered with *O. sativa* and NERICA unlike in other reports (Ren *et al.*, 2003; Brondani *et al.*, 2003). When primer 873-H was used by excluding all other primers *O. glaberrima* clustered with wild-Gambella and wild Gonder-2 (data not shown).

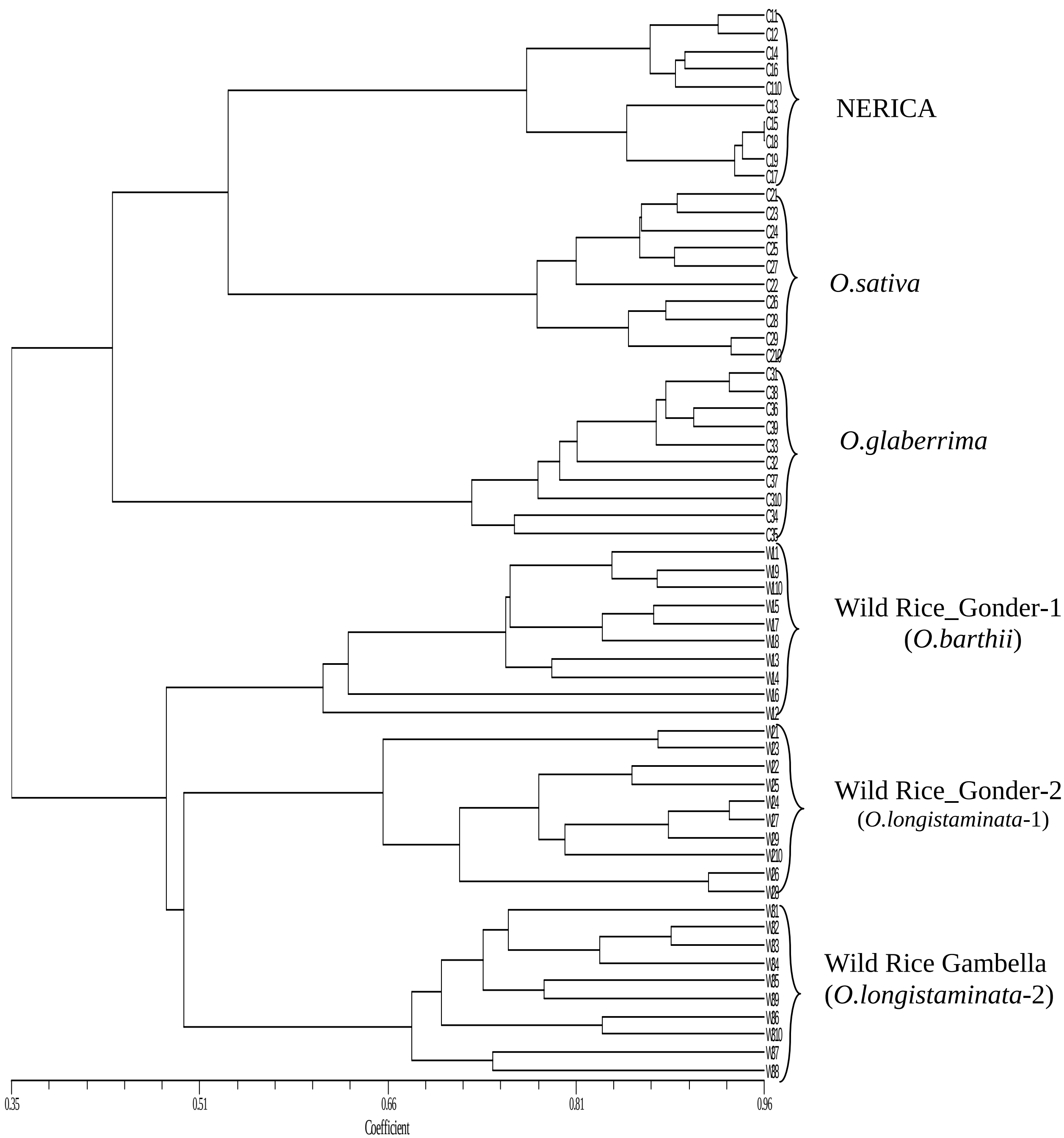
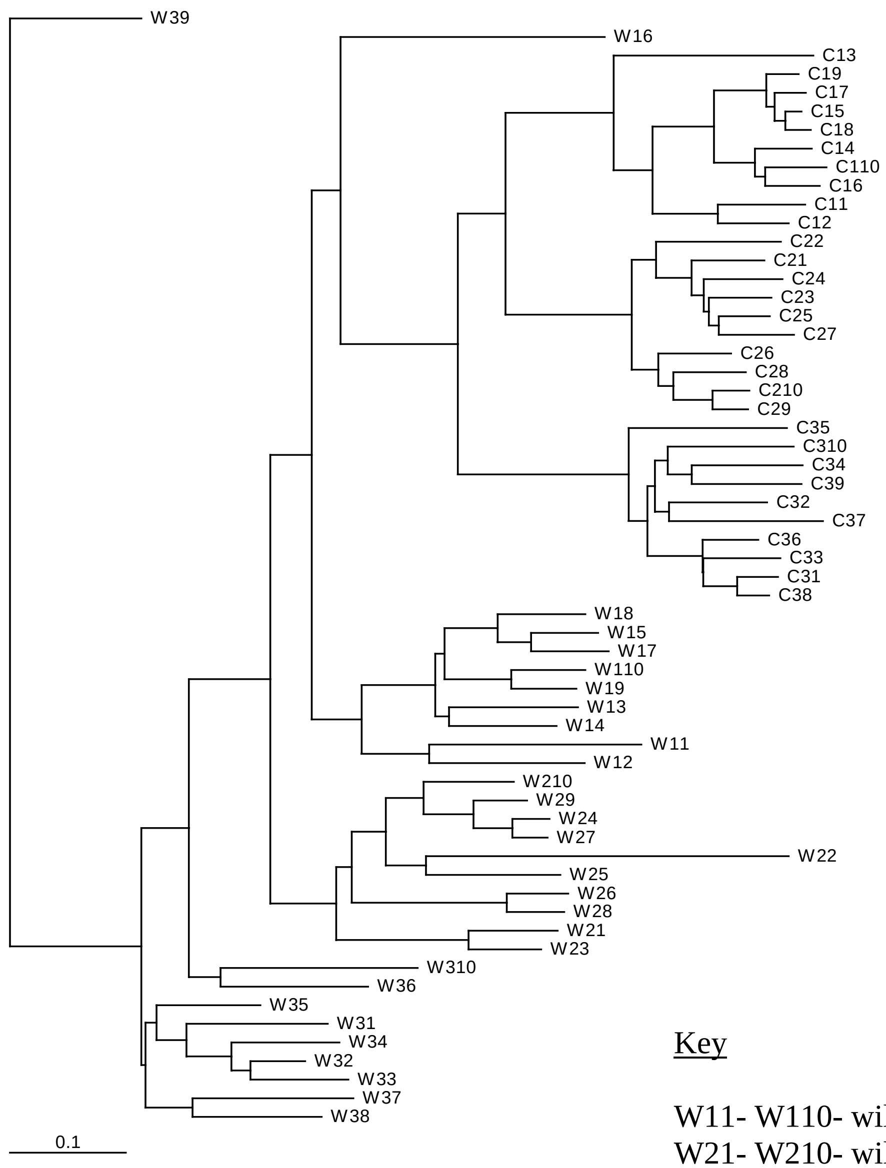


Figure 5.3:- Dendrogram for 60 individuals of 6 rice populations obtained using the unweighted pair group method with arithmetic average (UPGMA) of 93 PCR bands amplified (presence absence data) by four di nucleotide (810,824,834 and 854) and two tetranucleotide (873 and 878) primers. The UPGMA algorithm is based on Jaccard's coefficients obtained after pair wise comparison of the presence-absence fingerprint.



Key

W11- W110- wild Gonder-1
W21- W210- wild Gonder-2
W31- W310- wild Gambella
C11- C110- NERICA- 3
C21- C210- *O. sativa*
C31- C310- *O.glaberrima*

Figure5.4: Neighbor-joining analysis of 60 individuals based on 93 PCR bands amplified by four dinucleotide (810,824,834 and 854) and two tetranucleotide (873 and 878) primers. The neighbor joining algorithm is based on Jaccard's coefficients obtained after pair wise comparison of the presence-absence fingerprint.

5.9. PCO Analysis

All the data obtained using 6 ISSR primers were used in PCO analysis using Jaccard's coefficients of similarity. The first three coordinates of the PCO having eigenvalues of 7.04, 5.23 and 4.10 with variance of 15.30%, 11.36% and 8.91% respectively (Figure 5.6) used to show the grouping of individuals using three coordinates. All the 6 populations observed to form separate cluster. However, one individual sample from Wild Gonder- 2 observed to be intermixed with wild Gonder- 1 populations. Using two coordinates (Figure5.5) almost similar result was observed like that of three coordinates.

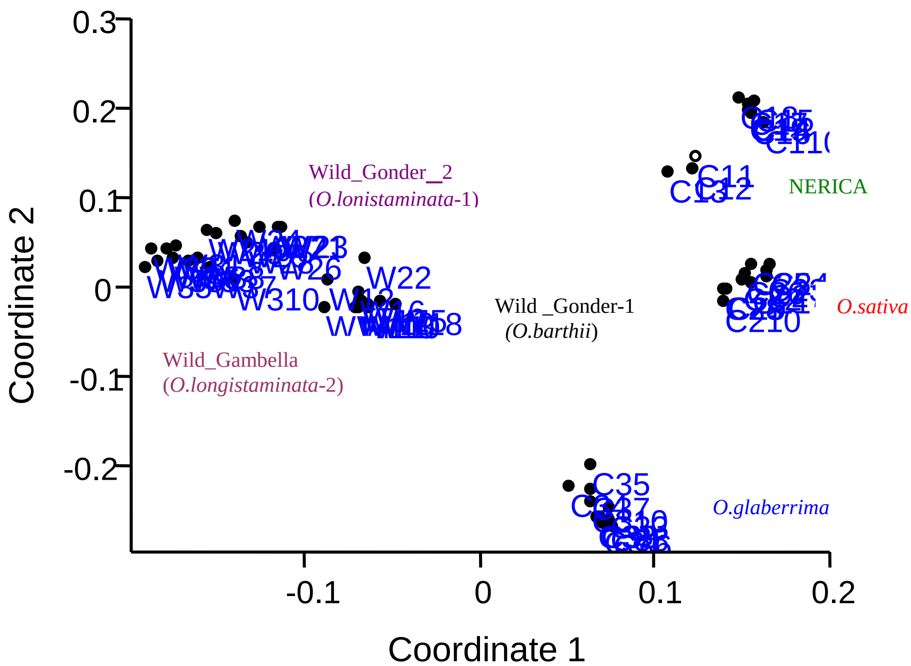


Figure 5.5: Two dimensional representations of 6 rice populations based on Jaccard's similarity coefficients.

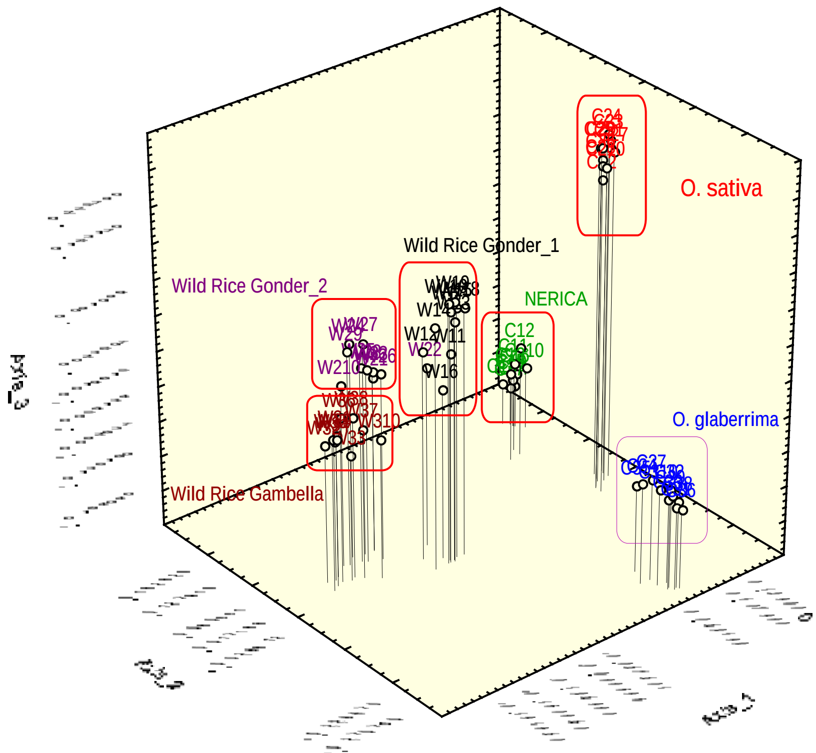


Figure 5.6:- Three dimensional representation of principal coordinate analysis of genetic relationships among 60 individuals of 6 populations of wild and cultivated rice inferred from similarity matrix using the Jaccard's index.

6. Discussion

6.1. Genetic diversity of rice

Generally, ISSR primers have high resolution power in fingerprinting and diversity analysis of rice (Joshi *et al*., 2000). In addition to the advantages (inexpensive, easy to generate), ISSRs are powerful in detecting polymorphisms. They can detect even more polymorphism than RFLPs in maize (Kantety *et al.,* 1995) and more than AFLPs in rice (Blair *et al.,* 1999). This result also confirms that ISSR marker is efficient in detecting polymorphism within and among populations and/or species of rice. In the present study, it suggested the existence of higher diversity among wild rice species in Ethiopia. Thus, ISSR marker systems will provide a useful tool in the future design of collection strategies for conservation and use of wild rice species in Ethiopia. Moreover, this marker observed to be very useful in detecting genetic diversity and population structure of Coffee (Aga, 2005; Tesfaye, 2006), Tef (Assefa, 2003) and Lentils (Fikru, 2006) collected from all over Ethiopia.

In the present study all the diversity parameters confirm that there is higher gene diversity in wild rice than cultivated rice species. Furthermore, wild Gonder-1 has been found to be a more diverse population as compared to other wild population studied. The comparative analysis of Asian cultivated rice (*O. sativa* ssp *indica* and ssp *japonica* with its close relatives; *O. rufipogon* and *O. nivara*) showed that the cultivars retained only 10% to 20% of the diversity in the wild species (Zhu *et al*., 2007). This is further observed in sunflower (*Helianthus annuus*), where only 40% - 50% of the diversity in the wild are maintained in the cultivars (Liu and Burke, 2006). Moreover, cultivated maize maintained approximately 80% of the diversity found in its wild ancestor (Wright and Gaut, 2005). The evaluation of SSR polymorphisms of sorghum indicated that landraces retained 86% of the diversity observed in the wild (Casa *et al.,* 2005). The drop in diversity is substantially greater for genes involved in domestication than neutral and random markers (Buckler *et al.,* 2001). The reason could be a sever bottlenecks during domestication and founding effect (small initial population) of crop cultivars.

O.glaberrima is observed to show higher percent polymorphism and Shannon's index as compared to other cultivated rice samples studied here. This could be either the seed is admixed or because of possible gene flow between wild rice populations in the surrounding areas. This can be further supported with presence of close relatives of cultivated rice, which are commonly found or coexist in rice farming system. Moreover, the AA-genome of weedy and wild relatives is highly compatible sexually with cultivated rice (Chen *et al.,* 2004). Hence, gene flow between wild and cultivated rice could be the main factor that could sometimes result in higher diversity of cultivars. This is further supported by Semon *et al* (2005) where the majority of accessions of *O. glaberrima* carry genetic evidence of admixtures due to introgression from *Oryza sativa*.

6.2. Distinction of wild verses cultivars

All the clustering analysis (UPGMA, NJ and PCOs) have clearly shown that Ethiopian wild rice are distinctly different from cultivated form of rice. Ren *et al.* (2003) with RAPD marker observed distinct group of the African *O. longistaminata* accessions and placed apart on UPGMA cluster from the Asian, Australian and other African group. But *O. barthii* and *O. glaberrima* accessions which are African wild and cultivated rice, respectively were randomly mixed with each other with relatively low variation since *O. barthii* is an immediate ancestor of *O. glaberrima* (Bautista *et al.*, 2001). In our result, however, *O. glaberrima* clustered with cultivars unlike the reports of (Ren *et al.*, 2003; Brondani *et al.*, 2003) which could be either due to the capacity of ISSR not illustrating true genetic relationship (Ren *et al.*,2003) rather complicated genetic variation or the seed used in this study might not represent the true African rice, *O. glaberrima.* The latter seems to hold since about 67% accessions of *O. glaberrima* studied are found to have admixed to genes of *O. sativa* (Semon *et al.*, 2005). *O. sativa* was introduced to West Africa by Arab traders and Portuguese navigators between 15 and 17th century. Since its introduction, it often grows in mixture with *O. glaberrima.* The present study supports the findings of Semon *et al* (2005) where the introgression of *O. sativa* into *O. glaberrima* germplasm are reported to have created intermediate types that cannot easily be distinguished at the phenotypic level from native cultivar of *O.glaberrima.*

Distinctions of wild genotype from cultivated were also evident in enset with RAPD profile (Birmeta *et al.*, 2004). The recent analysis by Tesfaye (2006) also showed a clear distinction of wild *C.arabica* from landraces and cultivars. Moreover, the neighbor joining tree analyses demonstrate long branch length of wild group which imply the existence of higher diversity in the wild gene pool. In sorghum, using SSR marker, it was observed that wild accessions from the same geographic origin tend to form the same group than distant locations and clustered by race. Moreover, the neighbor-joining analysis showed that wild sorghums generally formed a distinct group than cultivars (Case *et al*., 2005). However, the Ethiopian sorghum land race and wild sorghum revealed different pattern of clustering where samples collected from the same geographic area form different cluster based on RAPD data (Ayana *et al*, 2000) and the genetic diversity of wild sorghum is lower than that of landraces.

6.3. Genetic differentiation and population structure

Analysis of molecular variance (AMOVA) using RAPD markers for wild rice species showed less variation attributed to differences between individuals within a population and population difference within region (Ge *et al.*, 1999). In another observation using AFLP analysis of *Oryza* species, genetic variation increased from within species to between species (Aggarwal *et al.*, 1999). Similarly this result is also in agreement with the above RAPD and AFLP analysis. The AMOVA analysis showed highly significant (P=0.00) genetic differences among populations within groups, among groups (cultivated and wild) and within populations. Of the total variation 49.39% attributable to among populations within groups, 26.37% to among groups and the least, 24.24% to within populations. This is further supported with partitioning genetic diversity analysis of Shannon's and the majority of the genetic diversity (62.5%) reside among populations. The UPGMA and neighbor joining tree also confirm a clear grouping and differentiation based on populations of origin. The result is in line with the mating system of rice. Inbreeding species generally shows lower levels of diversity but greater interpopulational variation (Hamrick and Godt, 1990).

6.4. Relationships between wild and cultivated rice populations in Ethiopia

Wild rice species of Ethiopia and cultivated rice clustered separately and this confirms our understanding of traditional classification of *Oryza* taxonomy based on morphology and different molecular marker analysis (Joshi *et al*., 2000; Aggarwal *et al.*, 1999). Although *O.glaberrima* is not expected to form cluster with cultivars of *O.sativa* and NERICA since the wild populations used in this study are immediate ancestors of this species, due to admixturing of genes and introgression from *sativa* to *glaberrima* during the last 300-500 years of *O.sativa* introduction such clustering can result.

A cluster analysis (UPGMA and neighbor joining) was used to construct a dendrogram in order to see relationships among all individuals (Figure5.3 and Figure5.4). Each population forms a discrete cluster similar to the dendrogram constructed based on population of origin. Similarly PCO analysis based on two and three coordinates also show similar result except some observation in which a sample from wild Gonder-1 (*O. barthii*) intermixed with wild Gonder-2 (*O. longistaminata*-1) which might be due to their geographic proximity. In PCO analysis based on three coordinates wild Gonder_2 found between wild Gonder-1 and wild Gambella which might be a hybrid between the two wild populations of Ethiopia with more hybridized to wild Gambella stock. PCO also show better the hybrid nature of NERICA as a hybrid between the two cultivated rice species.

NERICA cultivar (NERICA -3), a hybrid developed through interspecific hybridization of the two known cultivated species of Asian and African origin (*O. glaberrima* and *O. sativa*) is expected to be intermediate between the two species, however, it is observed to be close to *O. sativa,* Asian rice, than the African rice species since it might shared most traits from its parent of Asian race due to successive back crossing of the hybrid to *O. sativa.* This fact is also indicated by Semagn *et al* (2006) that NERICA rice developed through F1 progenies derived from interspecific hybridization between *O.sativa* and *O. glaberrima* then backcrossed with their *O. sativa* parents.

The wild rice species collected from Gonder (wild Gonder-1 and wild Gonder-2) resembles more of *O. sativa* than *O. glaberrima* (Table 5.6); this could be because the samples are collected from an area where cultivated rice has been grown for the last 3 decades. The *O. sativa* used in this study was a variety named ‘X-jigna', which is the first rice variety released for south Gonder. Moreover, the cultivated and wild species are growing together as a crop and weed in the south Gonder. Hence, gene flow occurs with a noticeable frequency from cultivated rice to its weedy and wild relatives (Chen *et al*, 2004). In addition, among the wild rice species reported to be found in Ethiopia (Phillips, 1995), two of them (*O. longistaminata* and *O. barthii*) are with AA-genome and are sexually compatible with cultivated species having the same genome (AA). Their interspecific F1 hybrids could form complete pairing in meiosis thus resulting in gene flow between them. But in wild Gambella, since there is no cultivated rice near the vicinity where wild rice is found, a wild rice population from Gambella (wild Gambella) has less genetic similarity to *O. sativa* unlike the above case (Chen *et al.*,2004)

Wang *et al*. (1992), in their study on relationships in the genus *Oryza* using analysis of nuclear RFLPs, the cultivated African rice (*O. glaberrima)*, clustered with African wild species, *O. barthii*, suggesting that *O. barthii* is probably the ancestor of African cultivated rice, *O. glaberrima*. Wild Gonder-1 which resembles more *O. glaberrima* than the other two species (Wild Gonder-2 and Wild Gambella) in our result is *O. barthii*, which is immediate ancestor of *O. glaberrima* and a species from which African rice, *O. glaberrima* is originated (Bautista *et al.,* 2001). Wild- Gambella which has relatively distant genetic similarity with *O. glaberrima* is *O. longistaminata* and based on principal coordinate analysis, wild Gonder-2 which is found in between the two wild rice population in this study could be a hybrid between wild-Gonder-1 and wild Gambella with more back hybridized to wild Gambella stock. Moreover another wild species of rice, *O. punctata* (a species with two cytogenic groups:-BB, BBCC) which was genetically a bit further from cultivated rice is also reported to be found in Ethiopia (Joshi *et al*., 2000). However, collection, taxonomy and molecular phylogenetic analysis of wild rice in Ethiopia should be carried out to survey and confirm the occurrence and distribution of wild rice species.

It is also indicated by Phillips (1995) that in Ethiopia there is a genus *Leersia* in many parts of the country including where both cultivated and wild rice is being found. Within the tribe Oryzeae, where also the genus *Oryza* belongs, *Leersia* is most closely related to *Oryza*. Some species of both genera are even appearing to be intermediate between both genera (Vaughan and Morishima, 2002). Further detailed survey and studies are needed to delimit the species as well as occurrence and distribution of *Leersia* and *Oryza* genera found in Ethiopia so as to better understand their evolutionary relationships.

7. Conclusions

All the clustering analysis (UPGMA, NJ and PCOs) in this result have clearly shown that Ethiopian wild rice are distinctly different from cultivated forms of rice. Those wild populations collected from Gonder shows more affinity to *O. sativa*, which could be due to gene flow between wild rice since wild rice is growing as weed in cultivated rice farm while a population from Gambella shows less proximity to *O. sativa.*

In conclusion, through ISSR analysis, all the diversity parameters confirm that there is higher diversity in wild rice populations of Ethiopia. Furthermore, wild Gonder-1 (*O. barthii*) is found to be diverse as compared to other wild populations in Ethiopia.

8. Further recommendations and research needs

- This study is not exhaustive in terms of sample size and area coverage. Hence, more survey and sample collection has to be carried out so as to cover other agroecologies where probably wild rice occurs. Moreover, due attention should be given to areas where wild Gonder-1 is found since it is observed to harbor higher diversity than the other wild rice populations.

- Generally, these days, genetic diversity of wild rice populations in east and southern Africa is being eroded by the expansion of agricultural activities and overgrazing. Intensive efforts are, therefore, required to collect and conserve this diversity for future use in rice improvement programmes.

- Molecular phylogenetics and cytological analysis of wild rice in Ethiopia should be carried out to work out the distribution of wild rice species and their evolutionary relationship.

- Analysis with codominant markers system, like microsatellites, needs to be conducted to better understand and estimate the gene flow, the size of a population and levels of inbreeding.

- Further study is needed on occurrence and distribution of genera related to *Oryza* like *Leersia* in Ethiopia. Evolutionary relationships of the two or more genera coupled with crossability study, should be conducted to better understand the evolution of the related genomes and make use of *Leersia* and/or other genera to improve rice.

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