GENE FLOW BETWEEN CULTIVATED RICE (*Oryza sativa*) AND THE WILD RICE (*Oryza longistaminata*), AND ITS POTENTIAL ECOLOGICAL CONSEQUENCES IN KENYA

By

JAMES IRERI KANYA
B.ED (Hons.), (Kenyatta University), M.Sc., (JKUAT)
SCHOOL OF BIOLOGICAL SCIENCES
UNIVERSITY OF NAIROBI

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DECLARATION

This thesis is my original work and has not been submitted to any other university for award of a degree

JAMES IRERI KANYA ---------- 11/02/2010

Signature Date

This thesis has been submitted for examination with our approval as University of Nairobi supervisors

PROF. JENESIO I. KINYAMARIO ---------- 21/02/2010

Signature Date

DR. NELSON O. AMUGUNE ---------- 21/02/2010

Signature Date

DEDICATION

To my wife, Raphaera Ngera and our children; Dennis Kariuki, Fredrick Muchira and Felix Murithi and all ecologists.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABA	Abscisic acid
ABI	Applied Biosystems
ANOVA	Analysis of Variance
Bt	*Bacillus thuringiensis*
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EEO	Electro-endosmosis
FAO	Food Agricultural Organisation
GA	Gibberellic Acid
GE	Genetically Engineered
GM	Genetically Modified
GMO	Genetically Modified Organisms
HCL	Hydrochloric acid
ISB	Information Systems for Biotechnology
MIAD	Mwea Irrigation Agricultural Development
MT	Million Tonnes
NERICA	New Rice for Africa
NIB	National Irrigation Board
NRC	National Research Council
OECD	Organization for Economic Co-operation and Development
PCA	Principal Component Analysis
PIC	Polymorphic Information Content
RYMV	Rice Yellow Mottle Virus
SNK	Student-Newman-Keuls' test
SSR	Simple Sequence Repeats
TAE	Tris acetate-EDTA
TBE	Tris boric acid-EDTA
TE	Tris EDTA
TRIS	(Hydroxymethyl) aminomethane

UV	Ultraviolet
WARDA	West Africa Rice Development Association

ABSTRACT

Hybridization occurs in many crops that are sexually compatible with their wild relatives, leading to crop-to-wild pollen gene flow. Gene flow may result in evolution of super weeds, loss of genetic resources or even extinction of endangered species. In this study, gene flow between cultivated rice (*Oryza sativa*) and wild rice (*O. longistaminata*) was studied in Kenya.

For hybridization to be possible, dormancy breaking methods for *O. longistamianta* were investigated. Incubated and non-incubated hulled seeds were germinated in 1) Gibberellic acid (GA), 2) Hydrogen peroxide (H_2O_2), and 3). Water (H_2O) at two temperature regimes (31°C and 25°C). A similar set up for dehulled seeds was performed. Germination occurred in hulled but not in dehulled seeds. Highest germination percentage occurred in Gibberellic acid for all treatments. Significant ($P<0.05$) differences were observed within treatments but not ($P>0.05$) between treatments. No interaction effects ($P>0.05$) were observed between germination media and incubation and also with temperature. Highest germination was observed at higher temperatures (31°C).

Dispersal distance of rice pollen was investigated in Tana River district in the Coast Province of Kenya. Seeds of *O. sativa* were planted in a 50m diameter experimental design. Pollen traps placed at different heights (1.2 and 1.8 m) and distances (North, East South and West) from the pollen source were used. Pollen count decreased rapidly with increasing distance from the pollen source. Differences ($P<0.05$) in pollen count were observed in different directions but no difference ($P>0.05$) between the two heights. The highest pollen count was observed between 11:00 am and 12:00 noon.

F_1 hybrid seeds were generated from crosses between the two *Oryza* species under screenhouse conditions at Mwea Irrigation Scheme. Data on growth patterns, morphological traits and yield were scored in parents and hybrids. Only 6 % of the F_1 hybrid seeds set occurred with *O. sativa* as paternal plant but no seeds were formed in reciprocal crosses. Morphological differences ($P<0.05$) were observed among the plant types. In hybrids, yield correlated ($r=+0.843$) with flag leaf length but in *O. longistaminata* yield correlated with plant height ($r=+0.767$) and panicle length ($r= +0.664$). In *O. sativa*, yield correlated with plant height ($r= +0.741$) and panicle exsertion. ($r= +0.854$).

In order to confirm that the hybrids identified by morphological markers were true hybrids, molecular characterization was performed. DNA extraction and Polymerase Chain reactions (PCR) were conducted at the University of Nairobi. 22 rice simple sequence repeat (SSR) primers were screened to select markers that could identify the two parents and their F_1 hybrids. Six markers were found to be polymorphic across the samples. Polymorphic information content ranged from 0.223 for RM234 to 0.354 for RM280. A total of 12 alleles were detected and the number of alleles per primer was two. Through genotyping, F_1 hybrids were identified among the parents.

Generally, the main findings of the study were: 1) Dehulling of O. *longistaminata* seeds was found to be necessary for effective germination., 2) Hybridization can occur between cultivated rice (*O. sativa*) and wild rice (*O. longistaminata*), 3) To minimize such hybridization, a minimum isolation distance of more than 250 m between the two species is recommended, and 4) Use of morphology followed by molecular characterization is recommended for identification of F_1 hybrids in the field.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Cultivated rice is critical to the economies of many countries of sub-Saharan Africa (SSA), as it constitutes a primary commodity of agricultural development. There are two species of cultivated rice: *Oryza sativa* L. and *Oryza glaberrima* Steud. (Lu, 1999; Brondani *et al.*, 2002). The East and South African region is home to five wild rice species: *Oryza brachyantha, Oryza eichingeri, Oryza punctata, Oryza longistaminata* and *Oryza barthii* (Lu, 1999). In Kenya three species are reported: *O. punctata* and *O. longistaminata* which grow along the coastal region and *O. eichingeri* which grows in the western region (Vaughan *et al.*, 1991; Kiambi *et al.*, 2005).

The production of rice in Africa is compromised by losses due to shortage of water, pre-and post-harvest arthropod pests and diseases. To increase rural incomes and meet growing food demands, sub-Sahara Africa region must improve its agricultural productivity. The area is the only developing region where per capita food production has been declining and has the largest cereal deficits in the world (Wang and Ma, 2003). If there is no change in productivity, deficits will more than triple by 2020. Economists estimate that agricultural production in the sub-Sahara Africa must grow by 4% per annum during the next decade to stimulate a satisfactory level of general economic development (Brondani *et al.*, 2002; Wang and Ma, 2003). The current economic growth rate in SSA ranges between 1 and 2% (Wang and Ma, 2003).

The use of biotechnology has tremendous potential for providing genetic resistance to pests and diseases, which are especially the most problematic in the tropics (where most rice is cultivated) because of climatic conditions that are desirable to their year-round growth and reproduction (Brondani *et al.,* 2002). Small-scale farmers, representing many diverse cropping systems and growing environments, are typically least able to afford the means for combating biotic stresses, caused by pests and diseases, due to the high cost of pesticides. While some people may oppose the use of biotechnology in agriculture and aim to restrict the use of genetically modified (GM) crop varieties, biotechnology represents an opportunity to respond to urgent issues of hunger and malnutrition in Africa (Wu and Tanksley, 1993; Conway, 1997; FAO, 2004). The development of genetically modified (GM) rice with high uptake of iron and other essential minerals are examples of products that have the potential to directly improve the livelihoods of millions with limited resources.

It has been demonstrated that the wild relatives of rice species can harbour favourable genes that are not expressed in the phenotype of the landrace rice (Wu and Tanksley, 1993; Brondani *et al.,* 2002). For example, genes that enhance the yield characteristics of cultivated rice can be introgressed from wild relatives through genetic engineering (GE). This knowledge provides a strong case for preserving the wild species germplasm for future plant improvement using the new plant breeding techniques. Where a gene is expressed at some physiological cost to the plant, it will be eliminated from a wild population by natural selection, unless it confers a real advantage in the wild. Further, it should be acknowledged that the successful transmission of genes through pollen requires the presence of a sexually receptive partner (Thompson *et al.,* 1979).

The use of Genetically Modified Organism (GMO) for increased agricultural output has stimulated worldwide debate about biosafety assessment, intellectual property rights, international trade, and ethical issues (National Research Council (NRC), 2002). Like other crop genes, novel transgenes can spread through crop pollen and seed dispersal to populations of related crops, weeds and wild relatives (Song *et al.*, 2001; NRC, 2002; Ellstrand, 2003; Chen *et al.*, 2004). Many crops are sexually compatible with their wild relatives, leading to crop-to-wild gene flow in their sympatric regions (Ellstrand and Elam, 1993; Ellstrand, 2003; Snow *et al.*, 2005). Gene flow from crops to wild population may resort in evolution of the latter into super weeds or extinction of rare and endangered species (Ellstrand *et al.*, 1999).

Despite the lack of any demonstrated adverse effect of genetically engineered rice on human health (Delseny *et al.*, 2001), the possibility of some unanticipated effects cannot be ignored. In many crops, it is in the context of ecological stability and environmental impact that the use of transgenic resistant varieties should be evaluated. The possibility that transgenes will be transmitted to wild relatives growing in the vicinity of crop plants should be investigated due to the possible extinction of wild species in the natural ecosystems, loss of genetic resources and development of resistance to plant diseases and herbicides.

Transgenic crop varieties are now grown worldwide (Song *et al.*, 2006) and soon may freely be introduced in Kenya for commercial purposes, but before they are introduced certain biosafety issues need to be investigated. Several studies elsewhere have detected gene flow from cultivated rice to wild rice (NRC, 2002; Hauser *et al.*, 2003; Lexer *et al.*, 2003; Chen *et al.*, 2004; Whitney *et al.*, 2006; Song *et al.*, 2006).

However, no such studies have been conducted in Kenya and therefore, a better understanding of the cultivated rice-to-wild rice gene flow in Kenya is essential for ecological risk assessment.

1.2 Literature review

1.2.1 Taxonomic status of rice

The genus *Oryza* L. belongs to the tribe *Oryzeae* of the family Poaceae (Vaughan, 1994). There are 12 genera within the *Oryzeae*. The genus *Oryza* has 24 species of which 22 are wild species and two, *Oryza sativa* and *O. glaberrima*, are cultivated (Vaughan, 1994). There has been confusion in the literature concerning the correct nomenclature of the species most closely related to, as they often lack clear distinguishing morphological characteristics (Vaughan, 1994). However, recently attempts have been made to unveil this confusion. According to Vaughan *et al.* (2003) the genus *Oryza* has been classified into four complexes, based on their genetic diversity. The complex species are all diploid and have AA-type genomes (Table 1.1). Adaptation to different ecological niches has resulted in a continuum of morphological types from perennial to annual with intermediate varieties flowering more than once during a season. The species names presented in Table 1.1 have been adopted for consistency with the majority of the literature.

Table 1.1: The distribution and genomic classification of rice species throughout the world (Vaughan *et al.*, 2003).

Oryza species	Genome type		Africa	Central or South America	Asia	Oceania
complex						
L.	AA	24	√	√	√	√
O. glaberrima Steud.	AA	24	√			
O. barthii A. Chev.	AA	24	√			
O. glumaepatula Steud.	AA	24		√		
Chev.et Roehr.	AA	24	√			
O. meridionalis Ng	AA	24				√
O. nivara Sharma et Shastry	AA	24			√	
O. rufipogon Griff.	AA	24		√	√	√
***O. officinalis* complex**						
O. punctata Kotschy ex Steud.	BB, BBCC	24, 48	√			
O. malampuzhaensis	BBCC	48			√	
O. minuta Presl. et presl.	BBCC	48			√	√
O. eichingeri Peter	CC	24	√		√	
O. officinalis Wall ex Watt	CC	24			√	√
O. rhizomatis Vaughan	CC	24			√	
O. alta Swallen	CCDD	48		√		
O grandiglumis (Doell) Prod.	CCDD	48		√		
O. latifolia Desv	CCDD	48		√		
O. australiensis Domin	EE	24				√
O. brachyantha Chev. et Roehr.	FF	24	√			
O. granulata complex						
O. granulata Nees et Arn. Ex Watt.	GG	24			√	
O. meyeriana (Zoll et Mor. Ex Steud.) Baill.	GG	24			√	
O. ridleyi complex						
O. longiglumis Jansen	HHJJ	48				√
O. ridleyi Hook. F.	HHJJ	48			√	√
O. schlechteri Pilger	Unknown	48				√

Oryza sativa is the most widely grown of the two cultivated species. It is grown worldwide while *O. glaberrima* is grown solely in West African countries. Hybrids

resulting from the two species are replacing *O. glaberrima* in many parts of Africa due to their higher yields (WARDA, 1999). Study by Vaughan *et al.* (2003) has suggested that the progenitors are of the Asian species. These are *O. rufipogon* and *O. nivara*, which are perennial and annual respectively. *Oryza barthii* and *O. longistaminata* are thought to be the progenitors of the African cultivated rice, *O. glaberrima* (Oka and Morishima, 1967; Oka, 1988; Vaughan *et al.*, 2003). Other wild *Oryza* species are native to Africa, Central and South America, Asia and Oceania.

Unlike other cereals, domestication of rice represented characteristics not found in other major cereals. Principal among these is that rice evolved from the wild gene pool into two eco-genetic varietal types called *indica* and *japonica* (Oka, 1988). Majority of rice varieties in the tropics are *indica* whereas *japonica* is generally grown in temperate regions or at higher altitudes in the tropics (Dally and Second, 1990; Chen *et al.*, 1999). The two species differ in the type of chloroplast (Morishima *et al.,* 1992). Several studies have suggested that perennial type of wild rice is similar to *japonica* varieties and annual wild rice is similar to *indica* varieties (Oka, 1988). Thus it is now generally considered that *indica* and *japonica* varietal groups were derived from different domestication lineages in wild rice, rather than within cultivated rice.

The basic chromosome number of the genus *Oryza* is 12. *Oryza sativa* is diploid ($2n = 24$) representing the smallest genome (430 million base pairs) among the food crops. The genome is composed of repetitive sequences (Chang, 2003). Most other *Oryza* species are also diploid, but some are tetraploids ($4n = 48$). Genome symbols, A to F, are assigned to the species on the basis of meiotic chromosome pairing of F_1 hybrids. Those species with the same genome symbols show no significant disturbance in chromosome

pairing in their hybrids. Based on this, species in this genus can be classified into ten different genome types, AA, BB, CC, BBCC, CCDD, EE, FF, GG, JJHH and JJKK genomes (Vaughan *et al.*, 1991; Ge *et al.*, 1999).

1.2.2 Rice growing conditions

Rice grows in altitudes ranging from 0-3,000 m above sea level with an annual rainfall averaging 800-2,000 mm that are well distributed over the growing period especially under the rain fed situation. Soils for rice cultivation are varied as the climate regime to which the crop is exposed to, but texture ranges from sand to clay. The optimum temperatures are 20-38°C. Rice is a semi aquatic plant and the only major annual food crop (except cocoyam) that thrives on land that is water saturated or even submerged, during part or all of its growth (Ministry of Agriculture of Kenya; Crop Development Division Annual Report, 2004). A variety of water regimes are used, including unsubmerged upland rice (10% of total cultivation), moderately submerged lowland rice (irrigated, 45%, or rain-fed, 30%), and submerged rice (up to 6m of water, 11%, or floating, 4%). Rice can grow in a wide range of soil types including saline, alkaline and acid-sulphur soils (Oka, 1988; Takahashi *et al.*, 1991; Vaughan, 1994).

1.2.3 Origin and cultivation of rice (table of content)

Oryza sativa was first cultivated in south-east Asia, India and China between 8,000 and 15,000 years ago (Vaughan, 1994). *Oryza glaberrima* has been cultivated since approximately 1,000 BC (Joshi *et al.*, 1999). Current cultivation for *O. sativa* is worldwide, extending from latitudes 35°S (New South Wales and Argentina) to 50°N

(Northern China). Ninety percent of all rice is grown and consumed in Asia and it is grown from sea level to 3,000 m and in both temperate and tropical climates.

A large number of cultivars have been developed through centuries of rice domestication. The International Rice Gene Bank http://www.knowledgebank.irri.org) holds approximately one million different rice varieties (Vaughan, 1994). Cultivars can be distinguished on the basis of many characteristics, including the following; adaptation to different water regimes; growth habit and height; shapes, size and colour of the culm, leaf blade, panicle length and exertion, hull and grain size, degree of pubescence, disease and drought-tolerance (Takahashi, 1991).

1.2.4 Varieties of cultivated rice in Kenya

In Kenya, rice is popularly known by two trade names; the Pishori and Sindano. The two groups of rice have been grown in Kenya since the 1960's and their selection is based on the yield related traits and resistance to major pests and diseases (Kiambi *et al.*, 2005). Pishori includes all aromatic rice varieties such as Basmati 217 and Basmati 370 while the Sindano varieties are non-aromatic. The non-aromatic rice varieties in Kenya include IR 1561-228-3-3, IR 1529-167-2-2, BR51-74-6, IR54, BG-90-2, IR 2035-25-2, IR2793-80-1 and UP 254 (Table 2) (Ministry of Agriculture; Crop Development Division Annual Report, 2004).

Table 1.2: Characteristics of some of the rice varieties grown in Kenya (Ministry of Agriculture; Crop Development Division Annual Report, 2004).

Variety	Height (cm)	Maturity (days)	Yield (t/ha)	Cooking quality	RYMV	Blast
Basmati (Pishori) 217	118.0	122	4.6	Good	R	S
Basmati 370	118.0	122	5.3	Good	R	S
IR 2035-25-2	86.2	126	5.5	Good	MS	MR
IR2793-80-1	89.0	142	6.4	Good	S	S
BW 96	68.0	135	9.0	Fair	S	MR
UP 254	84.2	124	6.4	Good	MS	MR
AD 9246	78.2	128	5.1	Good	MR	MS

Key

R (Resistant); MR (Moderately resistant), S (Susceptible), MS (Moderately Susceptible)

1.2.5 Rice production

Rice is the second largest produced cereal after maize in the world (Rong *et al.*, 2006). Its production has been increasing since the beginning of the 1990s, with an annual production of 350 million tons (MT) and by the end of the century it had reached 410 MT and by the year 2006, the production was at 520 MT (FAO, 2009). Rice production is geographically concentrated in western and eastern Asia with more than 90% of world output (FAO, 2009). Other non-Asian countries that are most important in rice production are; Brazil, United States and Italy (Jahn *et al.*, 2000; Savary *et al.*, 2000; FAO, 2004). Africa accounts for less than 5% (20 million tonnes) of the global rice production (Vries and Toenniessen, 2001). Of this, West Africa predominates with over 65% and the rest (35%) comes from South and East Africa (FAO, 1999; Vries and Toenniessen, 2001;

FAO, 2009) (Table 1.3). In the latter, Tanzania takes the lead with over 80% of the rice production (FAO, 1999). The low rice production in Africa is attributed to poor water management, inadequate nitrogen and phosphorus levels in a wide range of soils and extensive use of low yielding local landlaces of rice (FAO, 1999).

Table 1.3: Average paddy production in Africa during selected periods (million tonnes; FAO, 2009).

Sub-region	1970s	1980s	1990s	2001–05	2006
West Africa	2.73	4.50	6.73	7.55	9.32
Central Africa	0.23	0.36	0.44	0.45	0.48
Eastern Africa	2.41	2.81	3.36	3.89	4.60
Southern Africa	0.12	0.11	0.15	0.18	0.20
Total	5.49	7.78	10.69	12.07	14.20

About 95% of the rice grown in Kenya is under irrigation managed by National Irrigation Board (NIB). The National Irrigation Board is a government of Kenya statutory board, which manages five rice schemes namely; Mwea Irrigation Scheme (in central Kenya), Ahero Irrigation Scheme (western Kenya), West Kano Irrigation Scheme (western Kenya), Bunyala Irrigation Scheme (western Kenya) and Tana Delta Irrigation Scheme (Coast Region) (Wanjogu and Mugambi, 2001; Ministry of Agriculture Annual Report, 2004). The remaining 5% of the rice (which is rain fed) is cultivated along the Kenyan coast, in Kwale, Kilifi, Lamu and Tana River districts and also in Busia and Teso districts of western Kenya. The main source of irrigation water is the river Tana for Mwea and Tana Delta Irrigation schemes and river Nyando for Ahero and West Kano Irrigation schemes from which water is either pumped or gravitated to the production areas. Although there is great potential for rice cultivation in rain fed ecosystems, only a

minimum proportion has been exploited (Ministry of Agriculture; Crop Development Division Annual Report, 2004).

In Kenya, rice is increasingly becoming an important foodstuff especially in the urban centres. Local rice production only caters for about 32% of the rice demand (Wanjogu and Mugambi, 2001). The average rice production per hectare under irrigation in Kenya is 5.5 tonnes for the aromatic varieties (Pishori) and 7 tonnes for non-aromatic varieties (Sindano). For rain fed rice the yield is about 1.5 tones per hectare (Ministry of Agriculture; Crop Development Division Annual Report, 2004). The irrigated area under rice in Kenya is about 13,000 hectares.

The area under rice in Kenya has continued to increase over the last six years, increasing from 10,781 to 15,940 hectares in 2005 (Ministry of Agriculture of Kenya, 2008) (Table 1.4). In 2006, the area under rice crop increased further by 45% to reach 23,106 hectares which resulted in rice production increasing by 12% from 57,942 tonnes in 2005 to 64,840 tonnes in 2006. The realized increase in production was attributed to Central Province where production increased from 43,192 tonnes in 2005 to 50,693 tonnes in 2006. However, in 2007, rice production declined by 27% from 64,840 tonnes in 2006 to 47,256 tonnes in 2007. This was as a result of decline in cultivated area which decreased by almost 29% over the same period (Ministry of Agriculture of Kenya, 2008). Rice production in 2007 represents a reversal from a consistent increase recorded since 2003.

Table 1.4: Rice production in Kenya in the period 2003 and 2007 (Ministry of Agriculture of Kenya, 2008).

Year	Production (Tonnes)
2003	40,498
2004	49290
2005	57,942
2006	64,840
2007	47,256

1.2.6 Diseases and pests

The common rice diseases are fungal (blast, brown spot, sheath blight, bakanae and dirty panicle), bacterial (bacterial blight and bacterial leaf streak) and viral (tungro, ragged stunt, gall dwarf, grassy stunt, orange leaf and the rice yellow mottle virus (RYMV) (Datta, 1981; Vries and Toenniessen, 2001). The RYMV is endemic to Africa, and affects lowland rice ecologies. Resistance to RYMV has been detected in cultivars developed from crosses of upland *japonica* and lowland *indica* rice varieties (Ndjiondiop *et al.*, 2005; Traore *et al.*, 2005). Blast is the most serious fungal disease of rice in Africa. It occurs throughout the continent in rice growing areas. Blast resistance has also been noted in developed varieties such as Katy, a variety developed in USAI (Lee *et al.*, 2005).

Blast fungus, *Pyricularia oryzae* Cav. is the most serious fungal disease of rice in Africa. In Kenya it accounts for 70-80% of the losses attributed to rice diseases (Wanjogu and Mugambi, 2001). The disease is more prevalent in the tropics due to favourable environmental conditions such as high temperatures (20°-30°C). The fungus also survives better in ecosystems with high relative humidity (92%) caused by the irrigation water, which is important for conidial germination and infection. Infestation is also accelerated

by availability of collateral host grasses (e.g *Setaria intermecia*) common in tropics (Vries and Toennniessen, 2001).

Birds and insect pests cause extensive damage to the rice crop in the field and to the grain during pre-and post-harvest. The brown planthopper (*Nilaparvata lugens*) and stemborers such as *Chilo suppressalis* cause serious damage to the rice crop. The most serious storage pests are the rice weevil (*Sitophilus oryzae*) and the lesser grain-borer (*Rhyzopertha dominica*) while *Quelea quelea* is the most dangerous bird pest to the rice crop in the field.

1.2.7 New Rice for Africa

Domestication of *O. glaberrima* took many years (about 3500 years) and therefore its ancestry and numerous generations of selection *in situ* led to successful adaptation of *O. glaberrima* in the African environment (Ndjiondiop *et al.*, 2005). On the other hand, Asian rice has been bred for intensive production and high yield, but outside of the African continent. The first Asian varieties arrived in Africa about 450 years ago, and they have subsequently replaced the local species over much of the rice-cultivated area. However, despite their popularity (as a result of their high yields), Asian rices are poorly adapted to many of the African environments where rice is grown (Ndjiondiop *et al.*, 2005; Vries and Toennniessen, 2001). New Rice for Africa (NERICA) is the product of interspecific hybridization between the cultivated African rice (*O. glaberrima*) and the Asian rice (*O. sativa*; WARDA, 1999).

Initially, crosses between the two species produced sterile progenies hence embryo rescue through tissue culture technique was necessary. The resultant plants were

then re-crossed (back-crossed) with the *O. sativa* parent to get fertile offsprings (often after several cycles of back-crossing) and the anther-culture was used to double the gene complement of male sex cells (anthers) to produce true-breeding plants (Vries and Toennniessen, 2001).

The new NERICA plants have vigorous early vegetative growth, a characteristic of *O. glaberrima* that gives rapid ground cover, followed by upright growth at reproductive stage. Rapid ground cover enables the rice crop to smother, and therefore out-compete weeds, while upright growth especially at reproductive stage, which is a characteristic of *O. sativa*, enables the plant to support heavy seed heads throughout maturity to harvest. The African species has weak flower- and seed-bearing stems which are prone to falling over (lodging) before harvest (WARDA, 1999).

1.2.8 Biology of wild rice

The term wild rice includes all the species of genus *Oryza* that behave as rice and which crop in rotation with rice weeds (Ferrero and Vidotto, 1998). Even though wild rice belongs to different species and subspecies, all these plants share the ability to shatter most of their grains immediately after ripening. Wild rice plants can also adapt to a wide range of environmental conditions. In most rice growing areas shift from rice transplanting to direct seeding has encouraged the spread of wild rice. This has become very severe over the last two decades after the cultivation of weak, semi-dwarf *indica*-type rice varieties (Diarra *et al.*, 1985; Engels *et al.*, 1995; Suh *et al.*, 1997). The spread has generally been favoured by planting of commercial rice seeds that contain grains of the weed. Wild rice grains frequently have a red-pigmented pericarp and it is for this

reason that the term 'red rice' is commonly adopted in international literature to identify these wild plants (FAO, 1999).

1.2.8.1 Wild *Oryza* species in Africa

1.2.8.1.1 Oryza brachyantha Cher.et Rhoer

This diploid (FF) African species is on the boundary of the genus Oryza. It is widely distributed across Africa but appears to be most abundant in West Africa (Vaughan, 1994). The species often grows with *O. barthii* in small temporary pools usually in laterite soils. This species has the smallest genome size in the genus *Oryza* 346 Mbp (Uozu *et al.*, 1997). Hybrids between *O. brachyantha* and elite rice lines have successfully been made to incorporate resistance to multiple races of bacterial blight (Brar and Khush, 1997).

1.2.8.1.2 Oryza eichingeri A. Peter

This is a diploid CC genome species of shaded habitats. It is adapted to wetter regions, average rainfall of 1275 mm per year. It is distributed in West and East Africa and Sri-Lanka (Vaughan, 1994). *Oryza eichingeri* is tufted non-rhizomatous habit, has flexuous awn, short non-split ligule and spikelet size. In common with *O. punctata* (BB) and *O. officinalis* (CC), *O. eichingeri* has 3 rDNA loci. However, one of these loci is much weaker in *O. eichingeri* than in other species of the *O. officinalis* complex (Fukui and Niizeki, 1983). *Oryza eichingeri* is widely distributed in Sri Lanka particularly the wet zone (Vaughan *et al.*, 2003). The species in Africa is morphologically similar to the one found in Sri Lanka.

Oryza eichingeri has been used as a source of resistance to brown planthopper in a rice breeding program (Yan *et al.*, 1997). The rate of success for inter-genomic crosses is very low (Sitch, 1990). Crosses between *O. sativa* and *O. eichingeri* gave a low out-crossing rate ranging from 0.36 to 1.65% (Yan *et al.*, 1997) and embryo rescue resulted in sterile F_1 plants. *Oryza eichingeri* is found in the western Kenya and the wet lands north-western of Tanzania.

1.2.8.1.3 Oryza barthii A. Chev.

Oryza barthii is widely scattered across Tanzania and in Ethiopia (Vaughan, 1994). The species is closely related to the African cultivated rice (*O. glaberrima*) and exhibit a strong annual life cycle, but unlike *O. glaberrima*, *Oryza barthii* is photoperiod sensitive. The species typically occurs in seasonally dry pools, has a short stature and large spikelets with a long strong awn (Bardenas and Chang, 1966). It can also occur in deep water where it shows a floating habit.

1.2.8.1.4 Oryza punctata Kotschy ex Steud.

Oryza punctata exists in two genomes, BB and BBCC with the two genome species showing morphological differences (Sano *et al.*, 1980; Sasahara *et al.*, 1982). It inhabits low altitudes and areas with higher temperatures compared to other species (Brondani *et al.*, 2002). The species has a similar distribution to *O. longistaminata*. Both species occur in Madagascar and across most of sub-Saharan Africa. The diploid race is more widely distributed in East Africa (Kiambi *et al.*, 2005) while the tetraploid race is more common

in central and West Africa. Among its ecological traits, shade tolerance was found to be high in the tetraploid form of *O. punctata* (Sasahara *et al.*, 1982).

The diploid race of *O. punctata* is a serious weed of rice. Due to the weedy nature of this species, it has been designated a noxious weed by the U.S. quarantine service. Hybrids between *O. punctata* and *O. sativa* have been reported but detailed analysis of these putative interspecific and intergenomic hybrids has yet to be conducted (Kiambi *et al.*, 2005). Although *O. punctata* has not been used in breeding programmes, there has been considerable effort to produce aneuploids of rice with chromosomes from *O. punctata* (BB) (Yasui *et al.*, 1992; Yasui and Iwata, 1996).

In Kenya the species is found in the coast Province; Kwale, Kilifi and Tana River districts. In Tanzania the species is widely distributed across the country from Dar es Salaam to near lakes Tanganyika and Victoria as well as central parts an the islands of Zanzibar and Pemba. The species also occurs in Uganda.

1.2.8.1.5 Oryza longistaminata Chev. et Roehr.

Oryza longistaminata is known by the following different names; wild rice, red rice or long-stamen rice. The species grows anywhere between about 40 and 2,100 m (Lu, 1999; Kiambi *et al.*, 2005) and occurs throughout tropical Africa and Madagascar (Gibbs-Russell *et al.*, 1989). The species is found in swampy areas, at the edges of lakes or ponds, streams or river sides, in water up to 4 m deep, but usually 1 m or less and grows in full sunlight in open savanna or openings in rain or galley forests (Gibbs-Russell *et al.*, 1989).

O. longistaminata is an erect, perennial hydrophytic grass with extensive, branched rhizomes that grow to a height of 2 m tall or more. Culms are spongy with auriculate leaf blades (450 mm long and 15 mm wide). The ligule is an unfringed membrane, 15-45 mm long and acute or 2-cleft, greater than 15 mm. The inflorescence is an open or loosely contracted panicle. The spikelets are 4.5-11.5 mm long, compressed laterally and disarticulating above the glumes. Spikelet-bearing axes are persistent. Lemmas are 3-9 nerved, entire, with awns 40-80 mm long. Palea is relatively long but narrower than the lemma. The anthers are 1.5-8.2 mm long (Gibbs-Russell *et al.*, 1989).

The species is the most widely distributed of the African *Oryza* species. It is readily distinguished from other AA genome *Oryza* species (*O. barthii* and *O. glaberrima*) by its rhizomes and the pointed bifurate ligule. It is the most difficult AA genome species to conserve *ex-situ* because it is partially self-incompatible and hence only produces abundant seeds when it grows in large stands *in-situ* (Sano, 1989; Khush *et al.*, 1990).

No hybrids have been confirmed between *O. sativa* and *Oryza longistaminata* or *O. barthii* and *O. glaberrima* as it develops an incompatibility system that makes it reproductively isolated from the two species (Morishima *et al.*, 1992). However, natural weedy hybrid populations between *O. longistaminata* and have been reported (Chu and Oka, 1970; Ghesquiere, 1985; Causse and Ghesquiere, 1991). There is a greater possibility of forming hybrids when *O. sativa is* the maternal parent than the reciprocal (Sano, 1989; Chu and Oka, 1970). Five ecotypes of *Oryza longistaminata* have been reported (Ghesquiere, 1986) across Africa;

a). Ecotypes isolated from cultivated rice

- *Oryza longistaminata* that grows in flooded areas as deep water wild rice
- *Oryza longistaminata* that grows in temporary pools with *O. barthii*

b). Ecotypes that are sympatric with cultivated rice

- Growing in vicinity of cultivated fields but not weedy
- In vicinity of cultivated fields and weedy
- Weedy scattered plants in cultivated fields or recent fallows

Compared to cultivated rice, the wild rice species are more numerous, longer and with more slender tillers, with leaves which are often hispid on surfaces and easy seed dispersal after their formation in the panicle (Diarra *et al.* 1985; Kwon *et al.* 1992; Suh *et al.* 1997).

1.2.9 Transgenic rice

Various methods for introducing new genetic variation into breeding lines for high rice production have been employed. Among them is the conventional rice breeding which relies on sexual crosses between related species. Despite great progress in this approach, some scientists believe that it is unlikely to be sufficient to meet rice demands due to increasing global populations, especially in developing countries (Conway, 1997; FAO, 2004; Lu and Snow, 2005). One approach to meet this shortfall is by developing transgenic crops.

Genes conferring particular traits have been successfully transferred into different rice varieties through transgenic techniques (Ajisaka *et al.*, 1993; Yahiro *et al.*, 1993; Liu *et al.*, 2000). Among the biological pesticides, toxins produced by bacteria such as *Bacillus thuringiensis* (*Bt*) have been the most successful in insect control where *Bt* gene

has been successfully introduced into rice. These bacteria produce insecticidal crystal proteins called endotoxins that are highly toxic to insects. Experimental lines of transgenic rice have also been developed with traits such as increased yield, high protein content, pharmaceutical proteins, high vitamin content, and high resistance to diseases, insects and herbicides among others (Jia *et al.*, 2004). The possibility of disseminating transgenes and conferring resistance and other traits through pollen flow is particularly a major concern as discussed earlier.

In the USA, over 200 applications for experimental field tests of transgenic rice have been received since 1992 but transgenic rice has yet to be grown on commercial scale (Lu and Snow, 2005) due to its ecological risks. Many transgenic rice varieties are likely to be released for commercial production within the next ten years depending on the rates at which they are approved by regulatory agencies and then commercialized (Jia *et al.*, 2004).

1.2.10 Molecular markers

Molecular markers are useful tools for evaluating genetic diversity and determining cultivar identity. Compared to morphological analysis, molecular markers can reveal differences among various crops at the DNA level and thus providing a direct and reliable method of differentiating crops germplasm. Currently, different marker systems such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNAs (RAPDs), Sequence Tagged sites (STS), Amplified Fragment Length Polymorphism (AFLPs), Simple Sequence Repeats (SSR) or Microsatellites and Single Nucleotide Polymorphisms (SNPs) have been developed and applied to a range of crops

including cereals (Kottapalli *et al.*, 2006). When DNA profiling technology first came into use, restriction fragment length polymorphism was considered state-of-art (Wenz *et al.*, 1998; Tanksley and McCouch, 1997).

This was followed by random amplification of polymorphic DNA (RAPD), followed by Amplified Fragment Length Polymorphism (AFLP) and most recently use of microsatellite markers or simple sequence repeats (SSRs). A Comparison of the commonly used markers is summarized below (Table 1.5).

Table 1.5. Comparison of the commonly used markers (Gurta *et al.*, 1999; Semagn *et al.*, 2006).

Feature	RFLPs	RAPDs	AFLPs	SSRs	SNPs
DNA required (µg)	10	0.02	0.5-1.0	0.05	0.05
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
Ease of use	Not Easy	Easy	Easy	Easy	Easy
Reproducibility	High	Unreliable	High	High	High
Cost per analysis	High	Low	Moderate	Low	Low

This information suggests that AFLP and SSR markers are the most effective in detecting polymorphism because they are widely distributed within the genome (Gurta *et al.*, 1999). The two markers are reproducible and allow a quick scan of the whole genome for polymorphism within a large number of bands generated. However, SSRs are preferred to AFLPs because they are co-dominant with well known map positions in the rice genome unlike AFLPs which involve use of random dominant markers (Ni *et al.*, 2002).

1.2.11 Justification

Rice is one of the most important cereal crops, providing food for nearly one-half of the global population. It is also one of the earliest of the world's crop species to which transgenic biotechnology has been effectively applied for genetic improvement (Tyagi and Mohanty, 2000). With the rapid advance of transgenic biotechnology, more and more transgenic crop varieties are being released into the environment and entering commercial markets (Kym and Lee, 2006). The use of biotechnology in the development of transgenic crops has provided new opportunities for global food security and new developments in life sciences. However, release and use of transgenic products have caused tremendous concerns about biosafety (Kym and Lee, 2006; Ellstrand and Marshall, 1985). The potential ecological risks associated with transgenes escape through gene flow (or cross- pollination) are foremost among these concerns that need to be addressed.

When alien transgenes escape to, and express normally in wild relatives of transgenic crop species, they may persist and disseminate within the wild population through sexual reproduction or vegetative propagation (Ellstrand and Marshall, 1985). If the transgenes are responsible for resistance to biotic and abiotic stress such as drought, diseases and salt tolerance that can significantly enhance the ecological fitness of wild populations, the escape of these transgenes will probably cause ecological and agricultural problems such as producing aggressive weeds. Such weeds might get out of human control and result in unpredictable damage to local ecosystems. Sometimes the aggressive spreading of the hybrids with better ecological fitness could even lead to the extinction of endangered wild species in local ecosystems (Snow and Moran, 1997).

Therefore a better understanding of hybridization, including its frequencies and directions between crops and their wild relatives will facilitate the effective management and safe use of transgenic crops. It is widely assumed that transgenes introduced into modern rice cultivars such as *Bt* genes will make their way into wild rice populations.

The main issues addressed in this study are; determination of germination ability of *O. longistaminata*, dispersal distance of the rice pollen grain, assessment of hybridization potential between cultivated rice and the wild rice species. Morphological traits and molecular characterization using SSR molecular markers were used to determine the extent of hybridization. The work concentrated on cultivated rice (*O. sativa,* variety Basmati 370), generated through conventional breeding and obtained from the Kenyan coast. The study aimed at providing information on the potential hybridization between the two species and the ecological impact of gene flow in order to recommend appropriate isolation distances in the event that transgenic rice harbouring *Bacillus thuringiensis* (Bt) gene is introduced in Kenya.

1.2.12 Overall objective

To determine whether gene flow can occur between cultivated rice (*O. sativa*) and the wild rice species (*O. longistaminata*) found in the coastal region of Kenya.

1.2.12.1 Specific objectives

In order to test the hypotheses stated below the following specific objectives were formulated:

i). To determine appropriate germination conditions of the wild rice (*O. longistaminata*) seeds,

ii). To estimate the dispersal distance of cultivated rice pollen grains,

iii). To determine hybridization potential between *O. sativa* and *O. longistaminata* and carry out morphological characterization of hybrids and their parents, and

iv). To carry out molecular characterization of *Oryza sativa*, *O. longistaminata* and the F_1 hybrids and using SSR markers to establish the extent of hybridization.

1.2.13 Hypotheses

This study had the following hypotheses:

i). Physical and chemical treatments enhance germination of seeds.

ii). Rice pollen can be dispersed for distances.

iii). Hybridization occurs between cultivated rice (*O. sativa*) and the wild rice species (*O. longistaminata*), and that

iv). F_1 hybrids can be identified through morphological and molecular characterization.

CHAPTER TWO

EFFECT OF TEMPERATURE, INCUBATION AND EXOGENOUS TREATMENTS ON THE GERMINATION OF *Oryza longistaminata*

2.1 Introduction

Seeds of higher plants enter a state of development arrest known as primary dormancy before completion of the maturation process (Gardner *et al.*, 1985). Seed dormancy, is a condition that temporarily suspends visible growth of meristems and it is one of the factors that hamper germination of seeds of most plant species (Naredo *et al.*, 1998). Dormancy in seeds is an important trait in plants especially in wild species which inhabit some of the most unfavourable conditions. It is a complex character influenced by many genetic and environmental factors (Naredo *et al.*, 1998). In most species, dormancy varies considerably when seeds are harvested at various stages of ripeness (Roberts, 1961) and when stored at different temperature regimes.

Seeds lose dormancy more rapidly at warmer temperatures than in cooler temperatures. In nature dormancy is a highly advanced trait of the wild species, as seen by the ubiquitous presence in a large number of species from different taxa. It renders resistance to preharvest sprouting and prevents germination until favourable conditions prevail (Naredo *et al.*, 1998). For germination, quiescent or non-dormant seeds require only rehydration after release from primary dormancy while dormant seeds need additional external stimuli such as light, temperature, incubation and chemicals (Thomas, 1992; Kristie and Fielding, 1994).

Variation in seed dormancy has been reported in different species of wild rice (Chang and Yen, 1969; Seshu and Dadlani, 1991), and several studies have been

undertaken to break seed dormancy of cultivated rices (Ellis *et al.*, 1983; Rao, 1994). However, according to Vaughan (1994), the majority of wild rice species have strong seed dormancy. The author, however, recognizes that for most species there is no sufficient information to support this position although previous works (Takahashi, 1962; Cohn and Hughes, 1981; Das, 1985; Naredo *et al.*, 1998) indicate that wild *Oryza* species possess stronger seed dormancy than cultivated rice varieties. Some wild species of rice such as *O. meyeriana, O. granulata, O. longiglumis, O. ridleyi*, and *O. minuta* can only germinate under partial shade, while others grow well under full sunlight (Cohn and Hughes, 1981; Das, 1985).

Rapid and full germination of seeds is an essential first step towards effective utilization of rice germplasm and, therefore, their germination needs to be clearly understood. Limited literature on appropriate germination conditions of wild rice species and especially that of *O. longistaminata* is impairment to the utilization of this major wild rice species in Africa. Inadequate literature on the hybridization and out-crossing rates of this species is largely attributed to limited knowledge on its germination potential (Naredo *et al.*, 1998). In order to investigate hybridization between *O. longistaminata* and *O. sativa*, determination of some of the optimal conditions for dormancy breaking had first to be determined. In particular the study focused on (1) evaluation of the optimum temperature for germination of *O. longistaminata* and (2) determination of the most appropriate chemical and physical method of enhancing dormancy in *O. longistaminata* seeds.

2.2 Materials and Methods

About 1 kg of seeds of *O. longistaminata* was harvested from an experimental field (20 X 15 m) in Tana River district in the Coast Province of Kenya. The parental plants had been grown vegetatively for 7 months (from December to June 2006). The seeds were sun dried and placed in porous papers and transported to Nairobi where they were stored at room temperature (23-27°C) for 3 months. Chemical and physical dormancy breaking experiments were done as described by Naredo *et al.* (1998).

2.2.1 Viability test

Prior to germination test, viability tests were carried out on the seeds using tetrazolium salt solution (Cakmak *et al.*, 1993). Seven hundred and twenty (720) seeds of *O. longistaminata* were selected from the previously harvested seeds (section 2.2). Selection of the seeds was based on their morphology (i.e not physically damaged). The seeds were softened by soaking them in cold distilled water for 24 hours. They were then soaked in 1% tetrazolium salt solution for 1 hour at 40°C in the dark, after which they were washed several times with distilled water to remove excess solution. Seeds were considered viable when the embryo was completely stained red, orange or pink (Naredo *et al.*, 1998).

2.2.2 Dormancy breaking tests

Various dormancy breaking methods were tested in order to establish the best method of braking dormancy in *O. longistaminata* seeds. The methods tested included; 1) incubation and non-incubation of seeds at 50°C, 2) chemical treatments (Gibberellic acid

(GA), Hydrogen peroxide (H_2O_2) and water (H_2O), and 3) physical treatments; seed dehulling (removal of the grain coat; lemma and palea).

2.2.2.1 Seed incubation

The 720 seeds (see section 2.2.1) were divided into two sets of 360 seeds each. One set of seeds was placed in an incubation chamber at a constant temperature of 50°C for seven days while the other set was kept at room temperature (23 -27°C). After incubation, the treatments described below were performed.

2.2.2.2 Effect of dehulling and chemical treatment on germination

One hundred and eighty (180) seeds were randomly picked from the 360 incubated seeds and dehulled (removal of the seed coat; lemma and palea). The dehulled seeds were placed in 6 petri dishes (labeled 1-6) lined with two sheets of filter paper. The seeds were soaked for 48 hours in each of the experimental solutions as follows: - Seeds in petri dishes 1 and 2 were soaked in Gibberellic acid; 750ppm, petri dishes 3 and 4 in 1.0 M hydrogen peroxide and petri dishes 5 and 6 were soaked in water.

The petri dishes 1, 3, and 5; (one from a pair of each of the test solutions) were placed in a growing chamber with a constant temperatures of 25°C while seeds in petri dishes 2, 4 and 6 (replicates) were placed in a growing chamber with a constant temperature of 31°C and their germination was monitored over two weeks. A similar set up for the remaining 180 incubated hulled seeds (with intact seed coat) was treated likewise.

In addition, a set of similar number of non-incubated dehulled and hulled seeds was set up in related conditions and their germination monitored over the same period. A seed was regarded as germinated when the radicle had pierced the seed covering structures or had reached 2–3 mm in length in isolated embryos.

2.3 Data analysis

Analysis of Variance (ANOVA; Appendix 1) was carried out using the R statistical software version 2.6.1 (R Development Core Team, 2006). Means were separated by Student-Newman-Keuls' test (SNK). The analysis was based on a split-split plot design, with chemical media being the main plot, incubation the sub-plots and temperature the sub-sub plots.

2.4 Results and Discussion

No germination occurred in hulled seeds within 2 weeks even after extending the germination period for additional 7 days (one week). Instead the hulled seeds were attacked by a fungus (*Aspergillus niger*) (Plate 2.1) after 14 days, and no further results were reported for these seeds.

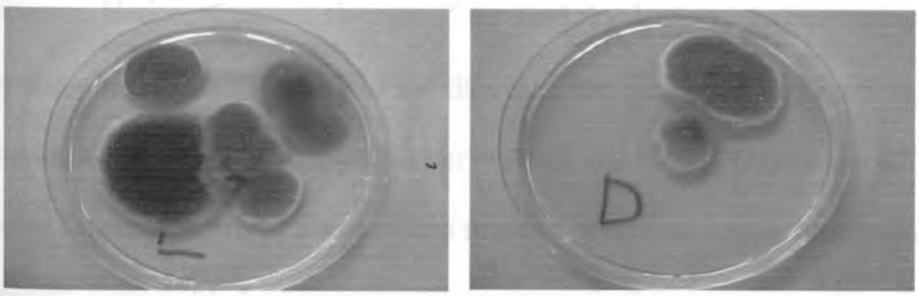

Plate 2.1: Hulled seeds of *Oryza longistaminata* attacked by *Aspergillus niger*

These results deviate from the findings of Naredo *et al.* (1998) who reported germination of hulled *O. longistaminata* seeds after extending germination period to about three weeks, in highly sterilized conditions. But even with that, germination occurred only when the hulled seeds were soaked in GA which reduces the seeds' sensitivity to low oxygen percentages and thus overcoming the inhibitory effect of the hull. In the present study, the attack of seeds by the fungus, *Aspergillus niger*, could possibly be attributed to contamination of seeds with the spores of this fungus.

The present study, however, recorded germination in dehulled seeds (Fig. 2.2) under other different experimental conditions. These results show that dormancy of *O. longistaminata* seeds is greatly associated with the seed covering structures. The results are in line with those of Bewley and Black (1985) who reported that rice dormancy is imposed in two common ways; by seed coverings (pericarp and testa) and the embryo itself. According to them, an intact (hulled) rice seed has a hard hull outside caryopsis that has an inhibitory effect on germination (Rao, 1994).

However, there are conflicting propositions on the effect of hull in rice seeds germination. Studies by Lenoir *et al.* (1986) suggest that seed covering structures prevent or reduce water imbibition and diffusion of oxygen which inhibits seed germination processes. Hence, structural changes of the seed that lead to increased porosity of the hull increase physical permeability of the tissues to the diffusion of oxygen and water which stimulates metabolic processes. Other studies on barley (*Hordeum vulgare* and oat (*Avena* fatua) seed grains also validate that the palea and lemma (that make the hull) are primarily responsible for imposition of dormancy in these species, as germination is much improved by their removal (Lenoir *et al.*, 1986; Benech-Arnold *et al.*, 1999).

Further, it is postulated that the pericarp and seed coat often contain phenolic compounds and alkaloids which inhibit seed germination (Bhattacharya *et al.*, 1999; Tao and Buta, 1986) and their removal facilitates germination. Indeed, phenolic compounds in the seed coat have been reported to function as germination inhibitors (Qi *et al.*, 1993) by blocking the activation of the oxidative pentose phosphate pathways which reduce disulfide bonds in the target molecules (Kobrehel *et al.*, 1992) leading to germination. Hull-and pericarp-imposed dormancy have also been documented in other cereal crops (Roberts, 1961; Seshu and Dadlani, 1991; Chang and Yen, 1969) and wild relatives of cereal crops (Takahashi, 1962; Wu, 1993; Gu *et al.*, 2002).

Other studies (Greipsson and Davy, 1996), however, have shown varying effect of hull on seed germination on different plant species. In some plant species such as *Leymus arenarius*, seeds with hulls germinated better than seeds without hulls; possibly because hulls protect seeds from drastic fluctuations in the moisture content of the soil (Greipsson and Davy, 1996). This implies that imposition of dormancy by the hull depends on the ecological and physiological fitness of each plant species.

For all treatment conditions (media, temperature and incubation), significant ($P<0.05$) differences were observed within the treatments but there were no interaction effects ($P>0.05$) of the three germination conditions. In addition, there were no interaction effects ($P>0.05$) between germination media and incubation temperature (Fig. 2.1). However, rate of germination significantly ($F_{[2,324]} = 72.167$; $P<0.05$) differed between GA and the other two media (H_2O_2 and H_2O) but no ($P>0.05$) difference in germination rate between H_2O_2 and H_2O. GA recorded the highest germination rate (80.5%) while water had the least germination (47.4%). Likewise, significant ($F_{[1,324]} =$

48.6; $P<0.05$) differences were observed between incubated and non-incubated seeds with incubated seeds recording generally higher germination rate than the non-incubated seeds in the three media (Fig. 2.1).

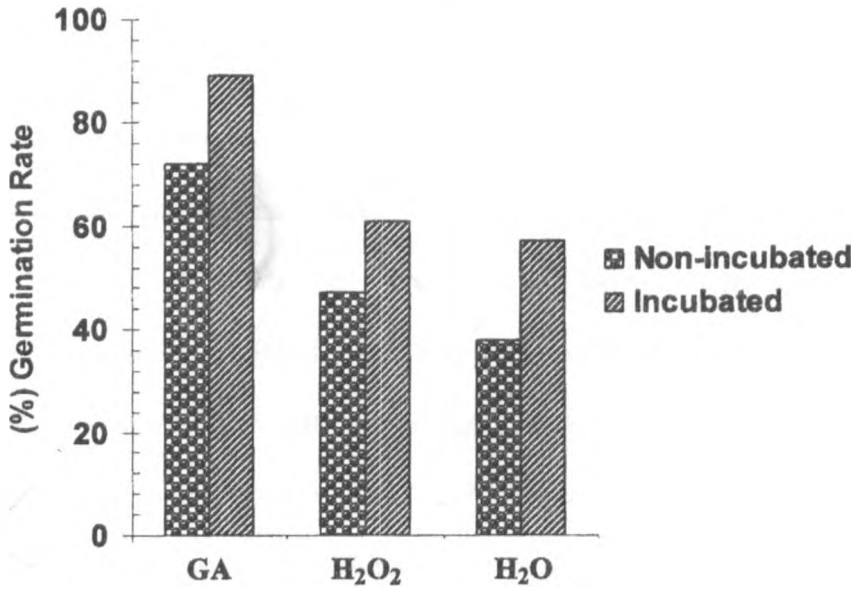

Figure 2.1: Effect of incubation (at 50°C) and germination media on germination rate of *O. longistaminata* seeds.

Lack of a biologically strong effect of the interaction of incubation and chemical media was surprising given that the two germination conditions are reported to play significant roles in dormancy breaking of the wild rice (Batlla *et al.* 2003; Huarte and Benech-Arnold, 2005). According to hydrotime model, incubation temperature is reported to influence the seed water potential. The model shows that as water potential becomes more negative, germination takes place under a wider range of water potential (Huarte and Benech-Arnold, 2005). According to the authors, if the water potential shifts

downwards, germination rate may increase. However, for the latter and according to the authors, there was an upward shift in water potential when seeds were incubated at 30°C. Similar observations were reported previously for lettuce (Dutta and Bradford, 1994) and potato (Alvarado and Bradford, 2002) seeds at supra-optimal incubation temperatures, and for *Orobanche aegyptiaca* seeds when incubation was performed at sub- or supra-optimal temperatures (Ekstam *et al.*, 1999). Therefore it appears that factors that govern changes in dormancy of seed populations, and those that terminate it (such as incubation, temperatures and GA), operate through a reduction of water potential.

There was no interaction effect ($P>0.05$) between temperature and chemical media, but significant ($P<0.05$) differences were observed between the types of media used. Equally, rate of germination was significantly ($F_{[1,324]} = 12.41$; $P<0.05$) different between the two temperature regimes with higher temperatures (31°C) recording the highest germination rate in all the three media (Fig. 2.2). In this interaction, GA still recorded the highest germination rate (85 %) in the higher temperatures.

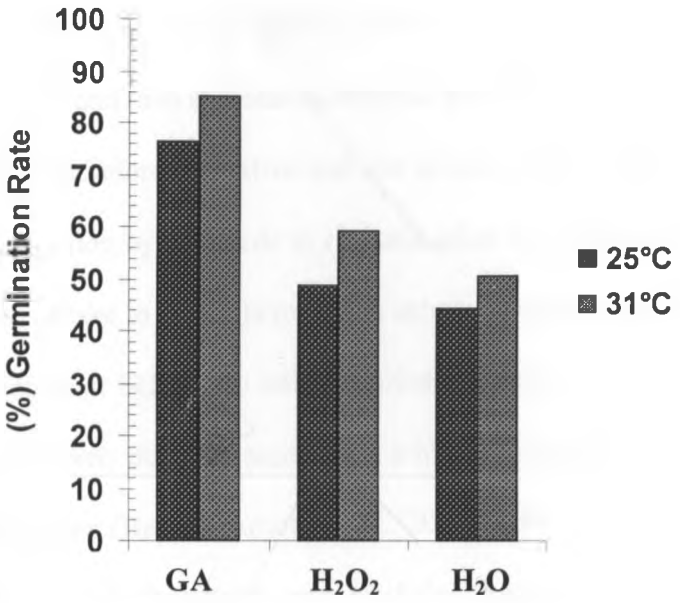

Figure 2.2: Effect of chemical media and germination temperature (25°C and 31°C) on percentage germination of *O. longistaminata* seeds.

These results are in line with the findings of Benech-Arnold *et al.* (1999) who found that the interaction effect of chemical media and temperature tended to reduce the individual effect of each treatment condition on seed germination. Their findings also revealed that GA acid greatly increased rate of germination of seeds compared to other germination media such as nitric acid and H_2O_2. Their findings however, indicate that for germination to occur, the balance between abscisic acid (ABA) and GA is critical as the two hormones have reciprocal effects on their respective biosynthesis and inactivation pathways (Seshu and Sorrells 1986; Gonai *et al.*, 2004). But, in most species of wild plants including wild rice, the mother invests the seeds with abscisic acid as a mode of adaptation (Takahashi *et al.*, 1991). Abscisic acid makes the embryo dormant until environmental conditions and native enzymes stimulate it to be broken down inside the

embryo. On the other hand, GA which is a naturally occurring plant growth regulator is synthesized in many seeds (Bewley and Black, 1985). GA induces hydrolysis of stored food reserves and thus stimulating embryo growth.

It is therefore indicative that use of GA is important in seeds dormancy breaking. Water imbibition by dry seeds in rice and other cereals is associated with a rapid increase in oxygen uptake to the embryo which enhances synthesis of abscisic acid (ABA) and GA by altering their balance to either promote or retard germination (Ogawa and Iwabuchi, 2001). However, dormant seeds have a higher content of ABA which decreases quickly after imbibition (Benech-Arnold *et al.*, 2006). ABA is one of the factors involved in rice dormancy and both natural and artificial dormancy breaking is proportional to the degradation of endogenous ABA (Hayashi, 1987).

Abscisic acid prevents cell wall from loosening by reducing their extensibility and increasing maximum turgor threshold for cell expansion (Schopfer and Plachy, 1985; Welbaum *et al.*, 1990). Analogously, the role of ABA in seed dormancy is to limit weakening of the structures surrounding the embryo, cell wall loosening, and radicle extensions which are the later events associated with dormancy breaking (Bewley and Black, 1985). On the contrarily, GA increases growth potential of embryo and overcomes the mechanical restraint conferred by the seed-covering layers by weakening of the tissues surrounding the embryo (Kermode, 2005).

Although the effect of interaction of H_2O_2 and H_2O with other exogenous factors had a lower mean germination compared to that of GA, the two solutions nonetheless also improved the germination rate. It has been argued (Ogawa *et al.*, 1996; Ogawa and Iwabuchi, 2001) that the breakdown of H_2O_2 results in increased O_2 level that enhances

oxidative respiration, which in turn promotes seed germination (Ogawa and Iwabuchi, 2001). However, in spite of H_2O_2 promoting seed germination, it has a high oxidative activity which makes it a toxic molecule (Ogawa and Iwabuchi, 2001) and is mainly generated under stressful conditions such as ultraviolet (UV)-radiations. Available literature (Asada, 1999; Ogawa and Iwabuchi, 2001) shows that excess H_2O_2 damages the plant as it impairs photosynthesis. But, exogenously applied H_2O_2 ameliorates seed germination in many plants (Chien and Lin, 1994). This could be attributed to solvent properties of water (formed by the breakdown of H_2O_2) in that it leaches out the phenolic compounds (germination inhibitors) in seed hulls which triggers germination signal by activating GA (Yaniv and Mancinelli, 1967; Toole, 1973).

In the present study, and compared to other two solutions, water had the least germination rates. This could be explained in that GA and H_2O_2 are produced by the plants and they act as plant regulators. Water activates hydrolysis enzymes that degrade storage protein into amino acids used for translocation of amylase enzyme. The enzymes in turn catalyze hydrolysis of starch to sugars that enhance respiration in the embryo triggering germination (Thompson *et al.*, 1979; Kristie *et al.*, 1981).

There was no positive gain in germination in the interaction between temperature and incubation. However, as stated above, germination rate between the two temperature regimes and between the incubated and non-incubated seeds were significant ($P<0.05$; Fig. 2.3). Rate of germinations for incubated seeds was higher (73.3 and 64.6%) compared to that of non-incubated seed (56.4 and 48.4%) in the two temperature regimes. This suggests that their interaction does not favour higher germination and that only the effects of the individual condition were realized.

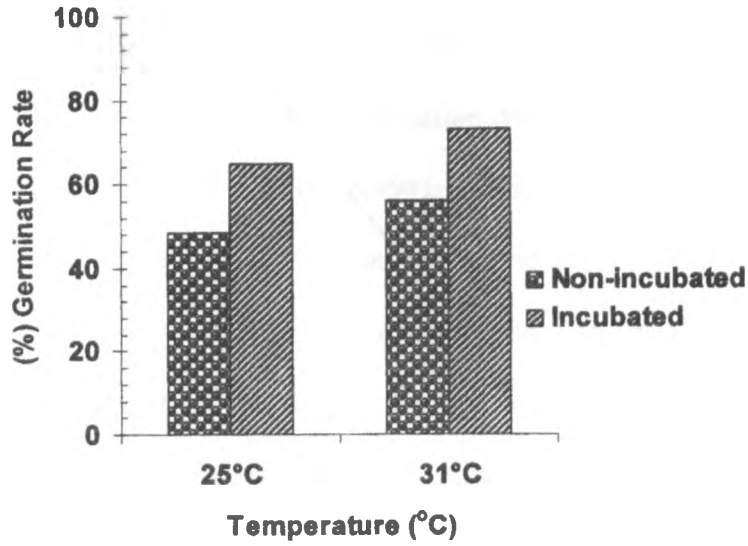

Figure 2.3: Mean germination of non-incubated and incubated *O. longistaminata* seeds in the two temperature regimes.

The influence of temperature on germination is in line with the findings of Kristie *et al.* (1981) and Doherty and Cohn (2000) who found that the rate of germination increased with increasing germination temperature. According to these authors, temperature has a major biological significance and that it affects most kinds of germinations including Phytochrome-mediated germination especially in some wild rice (Doherty and Cohn, 2000). A study of *Arabidopsis thaliana* found that Phytochrome has two photo-reversible confirmations: R light-absorbing Phytochrome (Pr) which is converted into FR light-absorbing Phytochrome (Pfr) by red (R) light and the far red (FR) light converts Pfr into Pr form (Shinormura *et al.*, 1996). This photo-conversion of Phytochrome by R and FR light regulates the onset of seed germination and it is also influenced by temperature.

In most cases, temperature influences Pfr level of total phytochrome as well as the conversion between Pfr and Pr in dry or imbibed seeds (Thompson *et al.*, 1979; Kristie *et al.*, 1981). The Pfr requirements for germination decreases with increase in temperature and according to Kristie and Fielding (1994), Phytochromes are synthesized rapidly and the conversion of Pfr to Pr also occurs quickly at higher temperatures (Kristie and Fielding, 1994). However, the influence of temperature on germination differs from one plant species to another as findings of various authors confirm (Noldin *et al.*, 1999; Gianinetti and Cohn, 2008). While in some studies (Huarte and Benech-Arnold, 2005) seed dormancy breaking is influenced by high temperatures, in others (Nam-Jin and Nam-Chon, 2003; Batlla and Benech-Arnold, 2003), it is influenced by low temperatures or even by alternation of low and high temperatures.

CHAPTER THREE

DETERMINATION OF DISPERSAL DISTANCES OF CULTIVATED RICE (*Oryza sativa* L.) POLLEN

3.1 Introduction

Rice is one of the most important cereal crops worldwide and the staple food for nearly one-half of the global population (Song *et al.*, 2003). It is therefore not surprising that it is also one of the first crop species to which transgenic biotechnology has been effectively applied (Tyagi and Mohanty, 2000), although commercialization of genetically modified (GM) rice has not begun. Many genetically modified rice varieties such as insect resistant (Bt) rice have been developed in confined environments (Rong, 2006).

However, cultivation of GM rice may have agronomic and environmental impacts that need to be evaluated and managed before their release and use (Lövei *et al.*, 2007). Pollen mediated gene flow is foremost among the impacts that need to be addressed (Lu and Snow, 2005). If the inserted genes, transgenes, spread to and are expressed in wild relatives of transgenic crop cultivars, they may persist and be disseminated within the wild population through sexual or vegetative propagation. If the transgenes are coding for resistance to abiotic or biotic stress such as drought, salt tolerance, pests and diseases, this might significantly enhance the ecological fitness of these wild or weedy populations. Specifically, this may lead to invasion of novel plant types, loss of biodiversity in natural ecosystems and production of aggressive weeds within agricultural fields (Ellstrand and Marshall, 1985; Raybould and Gray, 1993; Snow and Moran, 1997). If the inserted transgenes instead spread to neighbouring non-GM fields this may lead to

contamination of the harvested conventional rice seeds, which could affect marketing of these seeds due to contamination. This may be a problem especially in small-scale agriculture where distances between fields are small, and where seeds are recycled by farmers from year to year. Therefore, a better understanding of gene flow through pollen dispersal, including its frequencies, distances, and directions between rice and its wild relatives is necessary.

For transgenes to spread from a GM field and become incorporated into seeds in a neighbouring non-GM crop, several biological processes have to take place and be successful. Pollen should disperse between GM pollen donors and the recipient non-GM plants and successfully fertilize ovules. The resulting embryos further should survive and mature into full seeds. If transgenes are to be incorporated into the gene pool of a neighbouring wild population, some of the seeds and offspring plants carrying the transgenes further need to survive and reproduce successfully with other wild plants in the population. A first step in an impact assessment of gene flow from GM cultivars is therefore to evaluate the distances and directions travelled by pollen grains. Knowledge on pollen dispersal distances are of utmost importance in order to determine adequate isolation distances between GM, non-GM, and wild related populations. Isolation by distance is probably one of the most efficient ways of avoiding gene flow.

In this chapter, results of a study of pollen dispersal distances from rice fields in Kenya are presented. The work attempted to collect baseline information on the possible isolation distances between conventional rice fields and wild relatives (especially *O. longistaminata*) and the significance of time of the day and temperature on pollen dispersal.

3.2 Materials and Methods

3.2.1 Study site

The study was carried out in Tana Delta Irrigation Scheme, Coast Province of Kenya. The Scheme is located in the Tana River delta in the Coastal Province of Kenya, 250 km North-east of Mombassa (40°10' 55 E and 2°16'35 S). This site was selected because *O. longistaminata* is common in this region and the Scheme has adequate supply of irrigation water and that the large fields like this would be a likely first place to cultivate GM rice in Kenya.

3.2.2 Plant material and experimental design

Non-transgenic plant material (conventional species) was used since by the time the study was conducted, the Biosafety Act (2009) of Kenya had not been enacted to allow the use of transgenic material. Seeds of *O. sativa* (cv. Basmati 370) were obtained from the National Irrigation Board of Kenya, and germinated after soaking them first in water for 48 hours. The germinating seeds were then transplanted to a 2.5m x 2.5m nursery. After 22 days the seedlings were transplanted to the experimental field in a circular design with a diameter of 50 m. The spacing within and between rows within the circle was 15cm x 20 cm. A total of about 8,000 seedlings were planted.

To estimate the amount and direction of pollen dispersal, pollen trap method was used as described by Kearns and Inouye (1993) (Fig. 3.1 and Plate 3.1).

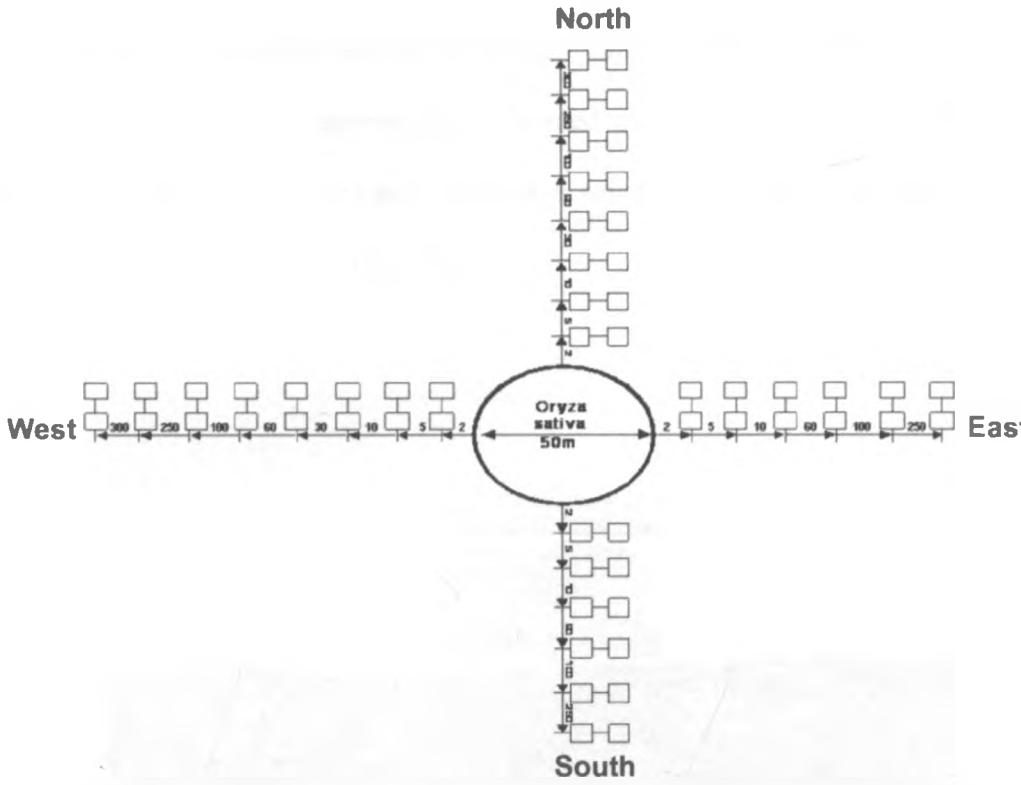

Figure 3.1: Experimental design showing arrangement of pollen traps at varying distances from the pollen source in a north-south and east-west directions.

Plate 3.1: Arrangement of pollen traps in different orientations of the pollen source (*O. sativa*).

Pollen was trapped on glass microscope slides (7.62 x 2.54 cm) coated with vaseline as bonding agent. Slides were attached to open-sided wooden plates (30 cm x 30 cm), with the adhesive surface facing towards the pollen source. Six slides were placed on each plate (together called a "trap"; Plate 3.2).

Plate 3.2: Pollen traps; upper (Up) placed at a height of 1.8 m and lower (Lp) placed at a height of 1.2 m, and fixed the glass slides (GL).

Traps for South (S) and East (E) were placed at intervals of 2m, 5m, 10m, 30m, 60m, 100m and 250m from the pollen source. For North (N) and West (W), which were the

main wind directions, the same arrangement of traps was used but with an additional one trap at 300m (Plate 3.2). This was to asses the effect of wind direction on pollen dispersal distances. At each distance, two pollen traps (upper and lower) were attached to a shaft, one at 1.2 m and another at 1.8 m above the ground. The two heights (1.2 and 1.8 m) reflected the average natural heights of *O. sativa* and *O. longistaminata* respectively. This was to mimic the natural heights of the two species so as to establish the plant species that would receive most pollen under the experimental conditions. One additional trap was placed at 2m distance to the N and W to assess diurnal pollen dispersal. For this, two traps were interchangeably used (when one trap was picked for pollen assessment, the second trap was erected at the same position to ensure continuity of pollen trapping) Fifty-nine pollen traps were used in total.

Data on pollen dispersal distances were collected within a period of 7 consecutive days during the peak flowering period in June 2007. During this period, pollen traps were placed daily from 8:00 to 17:00 hours. Flowering within a panicle in rice lasts for about 7 days (Sukhpal *et al.*, 2006) hence the choice of the flowering period. Temperature, humidity, wind speed and wind direction were measured during the pollen collection period using Microisis Systems-Agronic 4000-En 60947-1, England. In order to identify and differentiate the rice pollen from pollen of other plant species and specifically from those of grasses, reference glass slides of pollen were used for tallying (Plate 3.3). Pollen grains of each plant species are different from those of other species as they display unique sculpting of the pollen wall.

3.2.3 Estimation of dispersed pollen

The pollen traps were collected daily (at 17:00 hour) and taken to a confined space where pollen was counted using a light microscope. The glass slides were thoroughly cleaned, and kept in a glass slide container ready for use the following day. However, pollen traps for estimating the number of pollen produced per hour from the pollen source were collected after every one hour, pollen counted, and then returned to the same position (2m).

To assess the number of pollen grains caught in each trap, three of the six glass slides were randomly selected from each pollen trap and pollen grains were stained with aniline blue in lactophenol (Kearns and Inouye, 1993). Two cover slips, with their edges clearly marked for easy identification of the field of view, were randomly placed over the glass slides. Pollen grains (that were similar to those on the reference slides) within each cover slip were counted under microscope and their numbers recorded. For the identification of the pollen grains of *Oryza sativa* from other *Oryza* and grass species, a microphotograph of *Oryza sativa* (Os) (Basmati 370) pollen grain was used. Morphologically, rice pollen grains are initially spherical but collapse about 5 minutes after shedding from the anther (Plate 3.3)

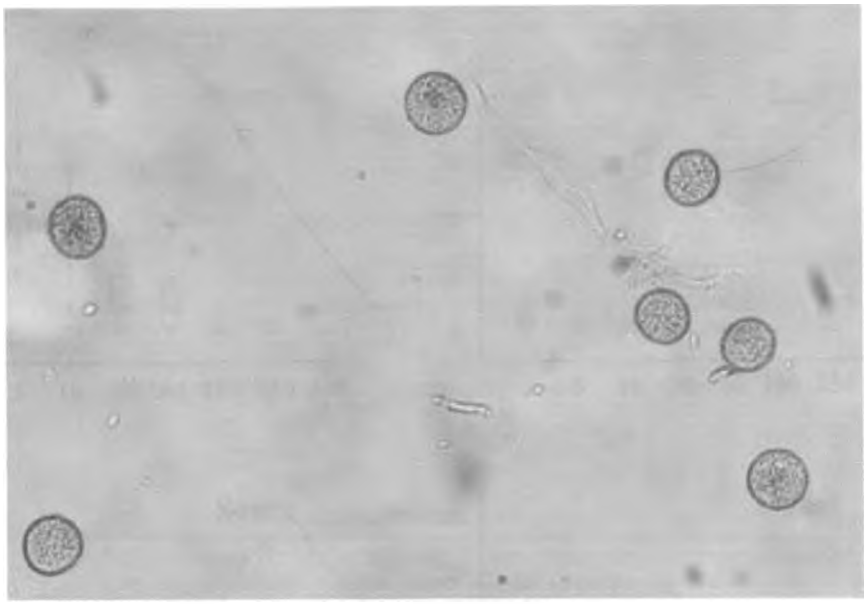

Plate 3.3: Reference slide showing pollen grains of *O, sativa.*

3.2.4 Statistical analysis

The relation between pollen counts on distance and direction were analyzed by Poisson regression while the effect of trap height was analyzed by Wilcoxon signed-rank, using the R statistical software version 2.6.1 (R Development Core Team, 2006).

3.3 Results and Discussion

During the six days of sampling, wind was mostly from the South with occasional and brief changes to the East. Most pollen was accordingly recorded on traps placed to the North while the least pollen was recorded in the South facing traps (Fig.3.1; significant differences between directions: poisson regression; $P<0.05$).

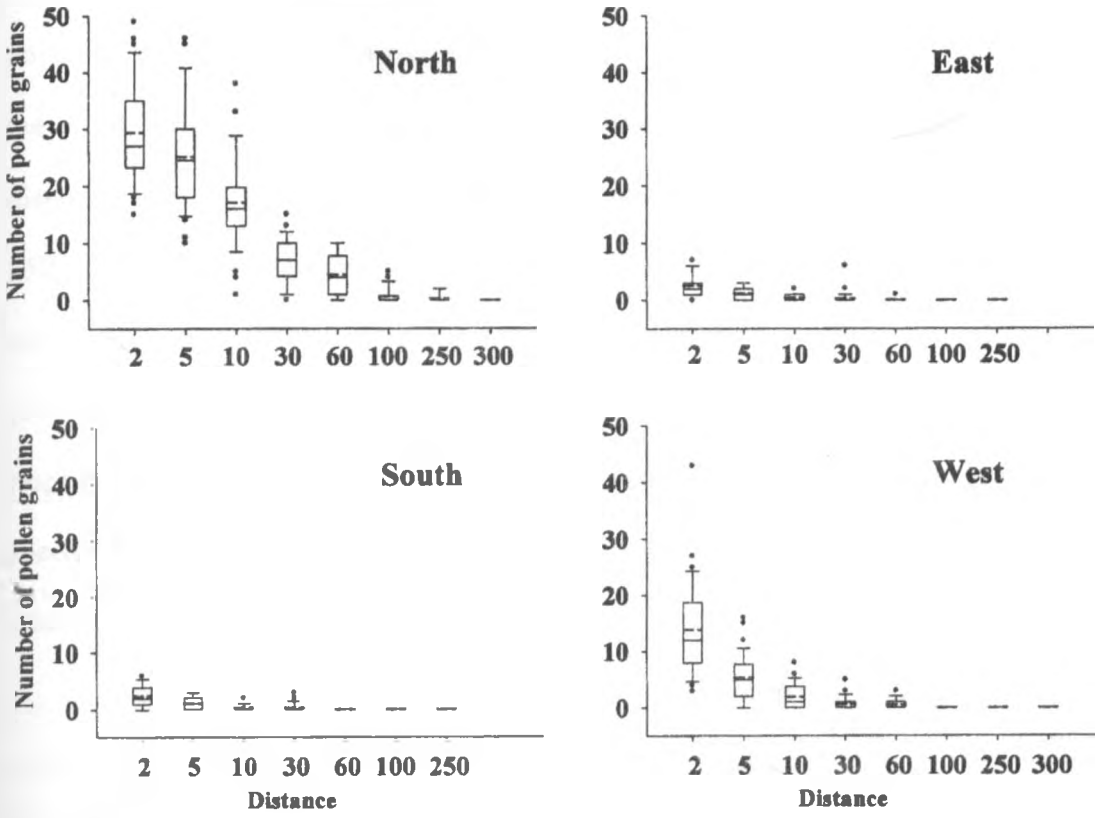

Figure 3.2: Number of pollen grains caught daily in the pollen traps, placed in different directions and distances from the source plot. In the figure, 25, 50, and 75 percentiles are *indica*ted by boxes, averages by broken lines, 10 and 90 percentiles by whiskers and more extreme counts by dots, respectively.

Wind direction had a significant effect on pollen dispersal with more pollen being dispersed to the north along the prevailing wind direction. The results are in line with a recent study by Hoyle and Creswell (2007) who showed that field-to-field windborne cross pollination varies greatly according to the wind direction. Wind speed has also considerable influence on pollen dispersal (Song *et al.*, 2004); in this study, wind speed was relatively stable (minimum 14 m/s, maximum 16 m/s.) and it could thus not be determined if pollen dispersal was affected by this.

There was a general decrease in pollen count with increasing distance. Most pollen was recorded within a range of 30 m. The furthest pollen was caught at 250 m to the North, and no pollen was recorded at distances 300 m in any direction. Pollen dispersal decreased significantly with increasing distance from the pollen source up to 250 m, and at 300 m distance to the North and West (the only directions in which pollen traps were placed at this distance). Other studies support these findings: Rong *et al.* (2007) recorded pollen at 2 m, and Wang *et al.* (2004; 2006) at 150 m and 250 m respectively from a pollen source. Although many of these studies do not indicate wind speed, Song *et al.* (2004) found the maximum distance of rice pollen flow to be 110 m when the wind speed was 10 m/s.

In this study pollen dispersal was recorded at 250 m when wind speed was 14-16 m/s, an indication that strong winds enhance pollen dispersal. Although 250 m distance may appear relatively short especially in large scale farming systems, in Africa and particularly Kenya, this is a long distance given that farming systems are of small scale types where farms are close to each other (about 50 m) and thus pollen flow from one farm to another is highly feasible.

The 250 m dispersal distance recorded in this study is however short in reference to pollen dispersal distance of other cereal crops such as maize (Messeguer *et al.*, 2006). Studies by Whitehead and Longman (1965) and Doebley and Iltis (1980) indicate that rice pollen grains are small (33-42 μm), and smaller than pollen of maize (58-99 μm; Oka, 1988); yet maize, which is wind pollinated like rice, disperses its pollen for longer distances (500 m) in field experiments and much further (32 km) in model studies (Doebley and Iltis, 1980). Although this comparison may not give the actual position of

rice pollen dispersal, since maize pollen is different from rice pollen, it can nonetheless be useful as a predictive reference for rice pollen flow. Furthermore pollen size alone may not be adequate for conclusive comparison of dispersal distance since other factors such as pollen density affect its dispersal. For instance, studies by Song *et al.* (2004) showed that the intensity of pollen flow (density) correlated with the size of the pollen source and therefore understanding pollen density would provide invariable information.

Other studies point out that pollen source size is important in dispersal of rice pollen; that the greater the size of pollen source (with the same plant density) the higher the pollen density detected at the same distance. According to Song *et al.* (2004), a pollen source field with a radius of 2.4 m recorded pollen dispersal distance of 19.2 m while pollen source field with a radius of 4.8 m recorded pollen dispersal distance of 24 m when wind speed was 1.72 m/s. Their results are in line with the present study which recorded pollen dispersal distance of 250 m with a pollen source field radius of 25 m when wind speed ranged from 14-16 m/s. In the present study, strong wind speed and large pollen source (with a radius of 25 m) were responsible for the relatively long dispersal distance although according to Rong *et al.* (2007), pollen source size is not a significant determinant of pollen flow.

It is generally understood that pollen dispersal is linked to height, that herbaceous species have more intensive pollen content at lower heights (Alcazar and Comtois, 2000), and that airborne pollen flow and source size are more significant than density, as is the case in anthophilous (influenced by feeding on flowers) pollen flow. However, the pollen flow of different plants differs significantly in their dispersal patterns (Alcazar *et al.*, 1998).

The study demonstrated varied movement of pollen grains at different heights. More pollen was recorded in the lower traps (1.2 m) than in the upper traps (1.8 m) within a distance of 2 m from the pollen source but, the opposite was true for distances further from the plot (Fig. 3.3), however, this was not significant ($P>0.05$).

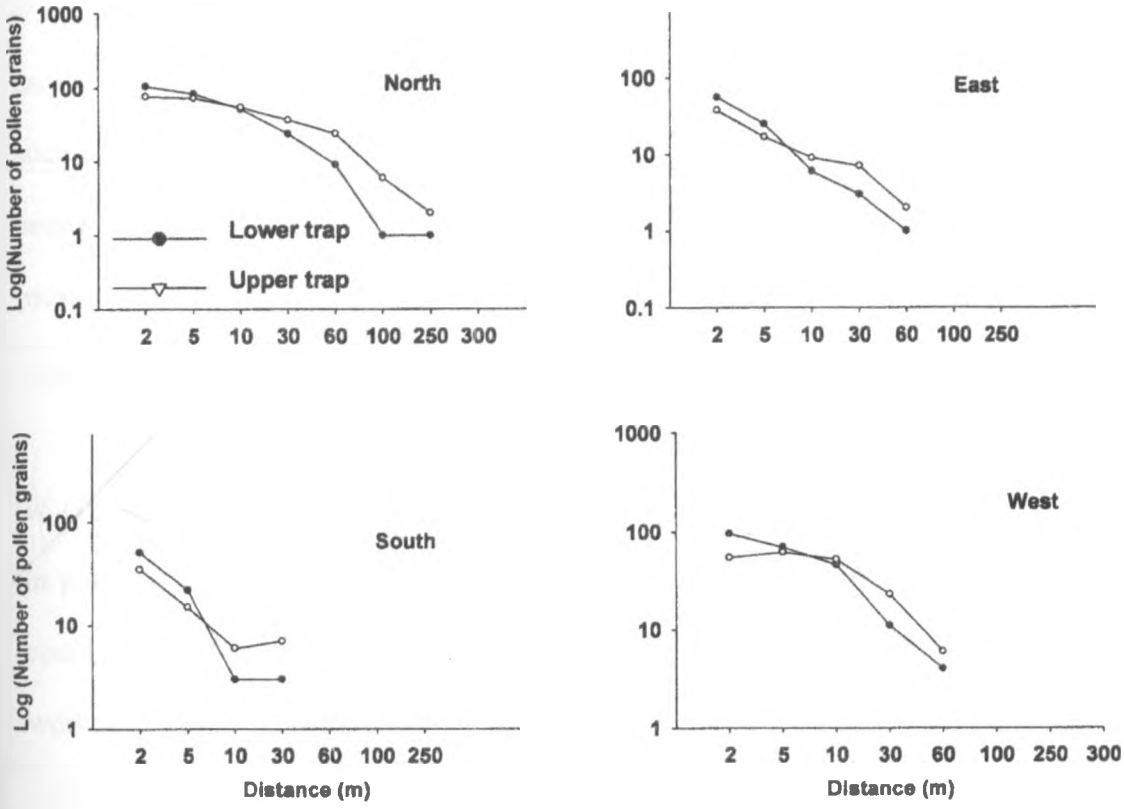

Figure 3.3: Sum of pollen grains trapped during six days in the upper and lower pollen traps, placed in different directions and distances from the source plot. No pollen was caught at distances without symbols.

The difference in pollen counts at different trap heights indicated that there might be differential outcrossing at the same distance from the pollen source for cultivated (*O. sativa*) and the wild rice species (*O. longistaminata*). Variation in pollen dispersal

between the upper and lower traps may have been caused by wind dynamics; lifting up the pollen near the pollen source and letting it fall before 300 m resulting in greater pollen density at the lower traps (1.2 m) close to the pollen source than at the upper traps (1.8 m). As distances increase from the pollen source, the wind gradient tends to favour the upper pollen traps (1.80 m). It is shown in the literature (Messeguer *et al.*, 2001; Rong *et al.*, 2006) that airborne pollen flow can follow an exponential leptokurtic pattern and can be greatly influenced by meteorological factors, especially microclimatic conditions, such as wind speed and direction, ambient temperature, and relative air humidity. The present results tend to differ from those of Song *et al.* (2004) which showed that movement of pollen grains at different heights was significant; more pollen grains being captured at the lower heights than at the higher heights at longer distances.

The findings by Song *et al.* (2004) were also in tandem with those of Alcazar *et al.* (1998) who found that the height placement of the pollen traps is related to efficiency in pollen grain capture. Reasons for divergence of this study from the previous studies could be attributed to the narrow height range; 1.2 m and 1.8 m. Previous studies have worked on greater height ranges e.g. from 0.0 m to 2.0 m. Although there was no significant difference between the two heights, the results suggest that relatively short species such as *O. sativa* may receive more pollen when close to the pollen source and that the tall species such as *O. longistaminata* would capture far-traveling pollen. The relatively few rice pollen grains detected at higher height levels and near the pollen source, also indicate that tall crops such as sugarcane can be used as an effective buffer crop to limit the dispersal of pollen when there is insufficient space for isolation between transgenic rice and its wild relative species or non-transgenic counterparts.

Most pollen was recorded between mid-morning (10-11 am) and mid-day (12 noon) for most of the days (Fig. 3.4).

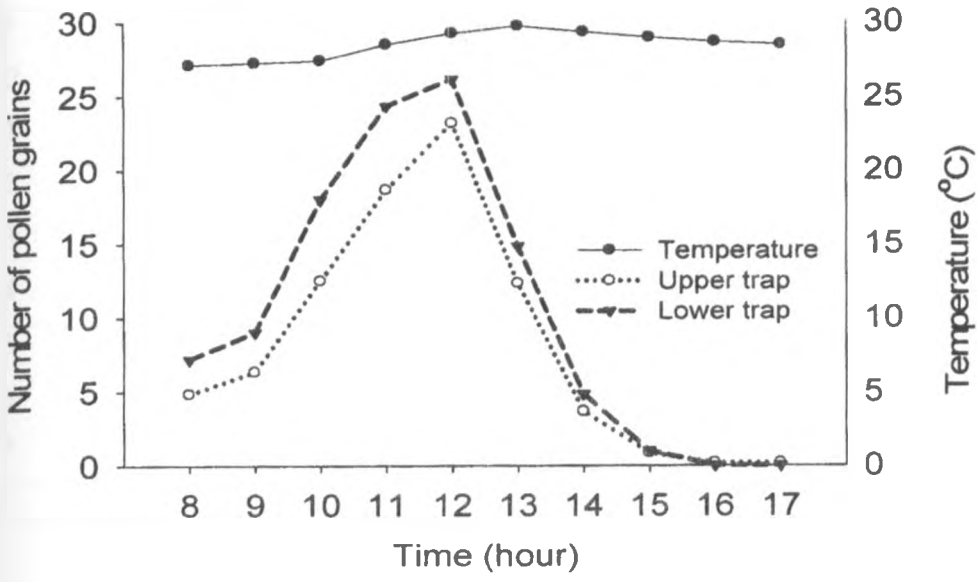

Figure 3.4: Average number of pollen grains caught in the upper and lower pollen traps from 8:00 to 17:00 hours during the six days sampling period. Traps were placed two meters from the source plot.

The highest pollen count was recorded at a mean temperature of 28°C and the lowest at 26°C. Wind speed and relative humidity were relatively stable during the experiment (14-16 m/s and 56-69%, respectively) and did not influence pollen dispersal ($P>0.05$) (Table 3.1).

Table 3.1: Hourly mean relative humidity and wind speed of six days, recorded from 8:00 to 17:00 hour.

Time (hour)	Relative humidity (%)	Wind speed (m/s)
8	69	14.2
9	69	14.1
10	69	14.8
11	65	15.2
12	60	15.8
13	59	16.3
14	58	16.0
15	57	15.4
16	56	15.7
17	56	14.8

Temporal factors had an influence on pollen dispersal. The high pollen counts between mid-morning (10-11am) and midday (12 noon) could be explained by the timing of floral anthesis. Rice florets are open from 9:00 to 15:00 hours depending on the rice cultivar and weather conditions (Datta, 1981; Khush, 1995). Although other authors (Horie *et al.*, 1992; Matsui *et al.*, 1999) have shown significant effect of temperature on pollen dispersal, in the present study temperature remained relatively stable (above 25°C) with minimal fluctuation of ±2 °C and therefore it was not possible to relate its effect on pollen dispersal. However, it has been shown that rice pollination is susceptible to temperature changes (Moon *et al.*, 2006). High temperatures (>35°C) at the time of flowering inhibit the swelling of the pollen grains, whereas low temperatures (below 16°C) impede pollen growth (Shimazaki *et al.*, 1964; Horie *et al.*, 1992). Since the driving force for anther dehiscence is the swelling of pollen grains at the time of floret opening (Matsui *et al.*, 1999), temperature stress reduces the percentage of anther

dehiscence at the time of flowering. In this study, most pollen dehiscence seems to occur at temperatures between 27°C and 29°C, below and above which (during the mornings and evenings) dehiscence significantly decreases. In this study the effect of humidity could not be assessed since it did not vary significantly (56-69%) between days.

In Kenya, about 95% of rice is grown in large irrigation schemes and only 5% is rain-fed (Ministry of Agriculture Annual Report, Kenya, 2004). The irrigated fields are divided into blocks for easy supply of irrigation water. The blocks are further subdivided into sub-plots which are allotted to individual farmers. Planting is synchronized per block. If GM rice is introduced into this production system, there might be a large pollen source available at the same time which could influence the probability of long distance pollen dispersal.

However, this would depend on whether all farmers would plant GM rice at the same time. Rain-fed farming is practiced in small scale farms (1-2 ha) in riparian habitats in the coastal region of Kenya. The type of farming coupled with inter-village seed exchange and the close proximity to wild rice populations (mainly *O. longistaminata*) would possibly encourage gene flow both between fields and to wild populations under the rain-fed production regime.

Typically the wild rice species are more outcrossing than the cultivated rice (Estorninos *et al.*, 2006). Various studies by Oka (1988), Oka and Chang (1961) and Song *et al.* (2003) indicate successful natural hybridization when *O. sativa was* the pollen donor to *O. longistaminata*. However, other studies by Noldin *et al.* (2002) indicate that pollen flow between cultivated rice and *O. punctata* can occur in either direction; often from the tall plants to the shorter plants than the reverse direction. This suggests that if

GM rice is introduced, hybridization would be greater in (tall) than in (short). Zhang *et al.* (2003) demonstrated that outcrossing rates between *O. punctata* and herbicide resistant rice (*O. sativa*) were higher (0.1 and 0.23%) with *O. punctata* as the pollen donor than when cultivated rice (*O. sativa*) was the pollen donor (0.0 and 0.14%) suggesting that gene flow will differ from crop-to-crop and from crop-to- wild.

CHAPTER FOUR

HYBRIDIZATION POTENTIAL BETWEEN CULTIVATED RICE (*Oryza sativa*) AND AFRICAN WILD RICE (*O. longistaminata*): CROSSABILITY, MORPHOLOGY AND SEED SET OF F_1 HYBRIDS

4.1 Introduction

Most domesticated (including genetically modified-GM) plants are able to spontaneously hybridize with wild relatives within their distribution range (Ellstrand *et al.*, 1999). Because of the possible impacts (such as development of aggressive weeds) of unintended gene flow, this subject has received much attention in the past years (Darmency, 1994; Snow and Moran, 1997; Spencer and Snow, 2001; Dale *et al.* 2002; Ellstrand, 2003; Wang *et al.*, 2004; 2006; Lövei *et al.*, 2007).

Asian rice (*Oryza sativa*) was introduced to East Africa by the Arabs when they settled at the East African coast about 6,000 years ago (Vaughan *et al.*, 2005). Cultivation of the species in Kenya was initially restricted to the coastal region but currently it has spread into the interior. In Africa, the Asian rice got in contact with several indigenous wild African rice species (see Chapter 1). Among these, *O. longistaminata* and *O. barthii* are likely to have hybridized with the introduced (Lu and Snow, 2005) as they share the AA genome. Both *O. longistamianta* and *O. barthii* occur in natural populations outside rice cultivation regions and as weeds in rice fields (Kiambi *et al.*, 2005). Under field conditions, *O. longistaminata* is believed to hybridize with

cultivated rice (Oka, 1961; Chu, 1970; Ghesquiere, 1985Kiambi *et al.*, 2005) but there have not been elaborate and well designed quantitative studies on this in Kenya.

Hybridization with the cultivated rice may have played a role in the evolution of weedy types of the wild species in African rice fields, as has been described for other weedy *Oryza* species (Lu, 1999; Brondani *et al.*, 2002). In Asia, the wild rice species such *as O. rufipogon* hybridize with cultivated rice resulting in hybrid plants mimicking the crop. However, hybridization in *Oryza* species depends on many factors among them, synchronization of the flowering time, outcrossing rate, occurrence of other species within the pollen flow distance and pre-and post-zygotic barriers such as the ability of the pollen to germinate on the stigma of the recipient plant and the eventual fertilization success.

To increase our understanding of the possible spread of genes, and among them transgenes, between cultivated and wild rice, this study aimed at investigating the following: 1) The extent to which cultivated rice (*O. sativa*) and the wild African rice *O. longistaminata* are able to hybridize under controlled pollinations, 2) Whether the growth and morphology of hybrids are distinct from the parents, which would allow the detection of hybrids in the field, and 3) If hybrids differ in their vigour and seed set compared to their parents.

4.2 Materials and Methods

4.2.1 Study site

The study was carried out in Mwea Irrigation Agricultural Development Centre (MIAD) located in the Central Province of Kenya, situated approximately 100 km North east of

Nairobi at the foot hills of mount Kenya (37° 20" E and 0° 40" S; altitude 1,159 m above sea level). The irrigation scheme covers an area of about 14,000 acres of paddy rice.

4.2.2 Plant materials

Seeds of *O. sativa* (Basmati 370) were obtained from the National Irrigation Board of Kenya while seeds of *O. longistaminata* were obtained from the Coast Province of Kenya as described in section 2.2.

4.2.3 Controlled hybridization

Twenty seeds each of *O. sativa* and *O. longistaminata* were germinated in 4 petri dishes as described in Chapter Two. Planting dates of seeds were staggered to harmonize flowering between the two species. The first set of seeds was planted in December 2006 while the 2nd and 3rd sets were planted at the beginning of January and February 2007 respectively. Seeds of *O. sativa* were planted in March 2007.

The resulting seedlings were transplanted into 6 plastic basins (3 basins for each plant type) with a diameter of 30 cm and a depth of 40 cm arranged in two blocks. In each basin, 20 seedlings were planted to make a total of 120 seedlings, 60 from each species. Fourteen seedlings in each basin were randomly selected and marked for further measurements of morphological traits and seed production per plant. At maturity (during flowering), 7 plants were randomly selected among the 14 marked plants from each basin for each species and were used as maternal plants. The other 7 plants were used as 'paternal' plants. From each of the maternal plants, all the florets (individual flowers) were emasculated by cutting off the tips of the florets

about one-third of the latter as described by Kaushal and Raven (1998) (Plate 4.1). The other 7 plants were used as pollen donors.

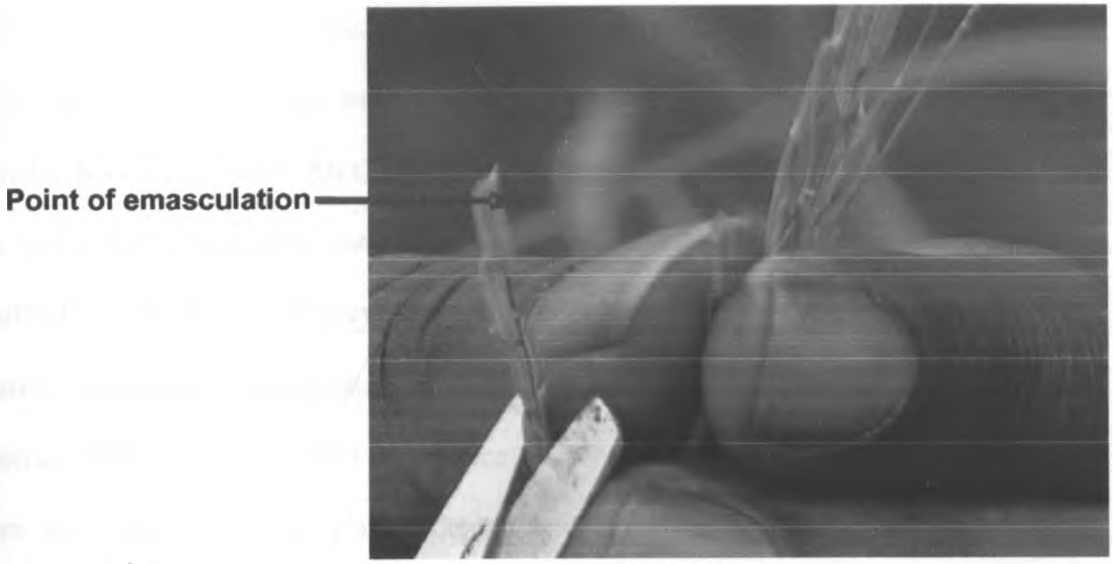

Plate 4.1: An illustration of emasculation of the flower (which becomes the floret).

The number of emasculated florets was arrived at as follows:-Each plant (genet) has an average of 9 tillers (ramets). Each tiller has one panicle, and each panicle has an average of 8 spikelets (one part of a compound inflorescence) with each spikelet bearing an average of 7 florets (Plate 4.4). Therefore in total, 280 florets were emasculated per plant. In each basin, 1,960 florets were emasculated (280 florets x 7 plants) and overall approximately 5,880 florets were emasculated in 3 basins for each plant species (280 florets x 3 basins x 7 plants). After emasculation, panicles from the other 7 'paternal' plants were cut off the following day at 8.00 hour and kept in separate flasks with warm

water for about two hours to facilitate opening of the florets as described by Kaushal and Raven (1998).

The recipient panicles were loosely covered with a porus paper (Appendix 2) to prevent any unintended pollen receipt. Once the panicles opened (about 10.00-11.00 hour), they were uncovered and pollen from *O. longistaminata* flowers was sprinkled over the maternal flowers. All tillers of an individual recipient plant were pollinated with the same donor, and only one donor was used. The porous paper was immediately returned over the panicle to prevent any further pollination from the other external pollen source. The process was repeated for *O. sativa* as the paternal and O. *longistaminata* as maternal and at the same time as that for reciprocal crosses. At maturity, the F_1 seeds were harvested from each plant, counted and stored in separate porous paper bags (labeled T_1-T_8). After 2 weeks, the seeds were incubated at 50°C for 7 days to break dormancy (see section 2.2.2.2). The F_1 seeds were divided into two halves. One half was germinated and the resulting plants were used for morphological characterization while the other half was kept in safe custody for later use in molecular characterization.

4.2.4 Measurement of morphological traits and seed production

Twenty seeds of *O.* sativa, O. *longistaminata* and F_1 hybrids were planted in the same experimental design as described above (section 4.2.3). Plant height was scored every two weeks from the time of radical emergence as the distance from the base of the stem to the tip of the longest leaf (Yoshida, 1981). Flag leaf length (Plates 4.2), panicle exsertion (the protrusion of the flower head) and panicle length were scored every 2 days

from the time of panicle initiation to the end of complete panicle formation (which took about three weeks).

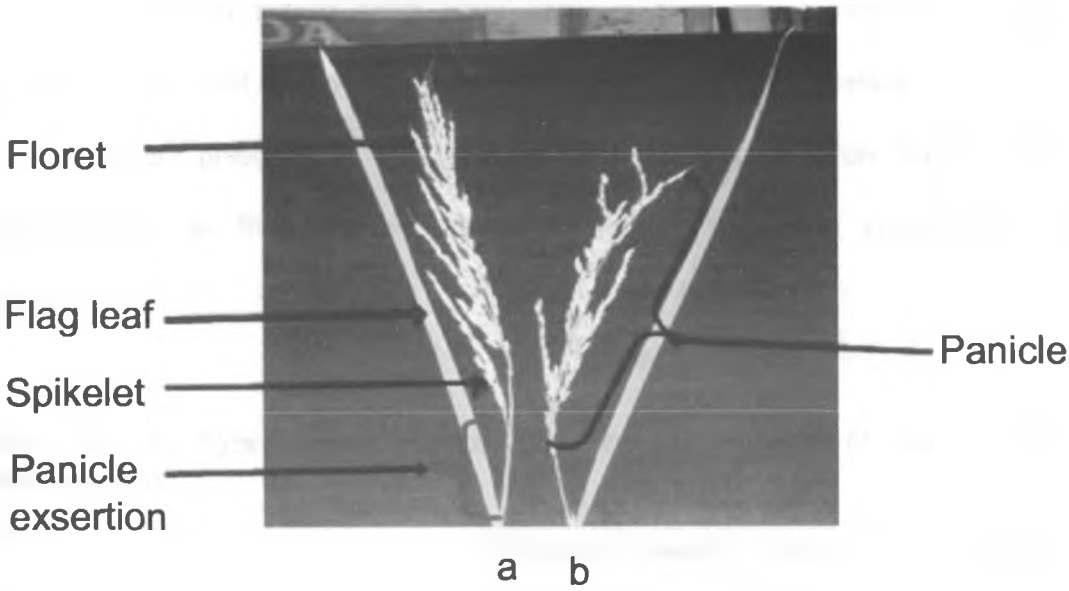

Plate 4.2: Flora morphological traits and flag leaves of (**a**) the F_1 hybrids and (**b**) *O. sativa*.

The number of spikelets per plant, awn (the terminal part of the bearded lemma) length, grain length and number of seeds were noted at harvesting.

4.3 Data Analysis

Morphological differences between cultivar, wild and F_1 plants were illustrated by box plots and analyzed by analysis of variance (ANOVA), using R statistical software version 2.6.1 (R Development Core Team, 2006). Student-Newman-Keuls' test was used to separate the means (Appendices 3-7). Morphological traits were additionally analyzed by

principal component analysis (PCA), using SPSS Ver. 12, (2007) to evaluate the combined ability of the morphological markers to identify hybrids.

4.4 Results and Discussion

A total of 330 F_1 hybrid seeds were obtained from the controlled pollinations, approximately 6 % of the potential seed number. All seeds were obtained with *O. sativa* as the recipient plant (Table 1; Plate 4.3), whereas no seeds were formed with *O longistaminata* as the recipient, despite seemingly successful emasculation and pollination.

Table 4.1: F_1 hybrid seeds formed from crosses between *O. sativa* and *O. longistaminata.*

Crosses		Number of florets emasculated	Number of F_1 seeds formed	Crossing success
Paternal	Maternal			
O. longistaminata	*O. sativa*	5,880	330	6 %
O. sativa	*O. longistaminata*	5,880	0	0 %

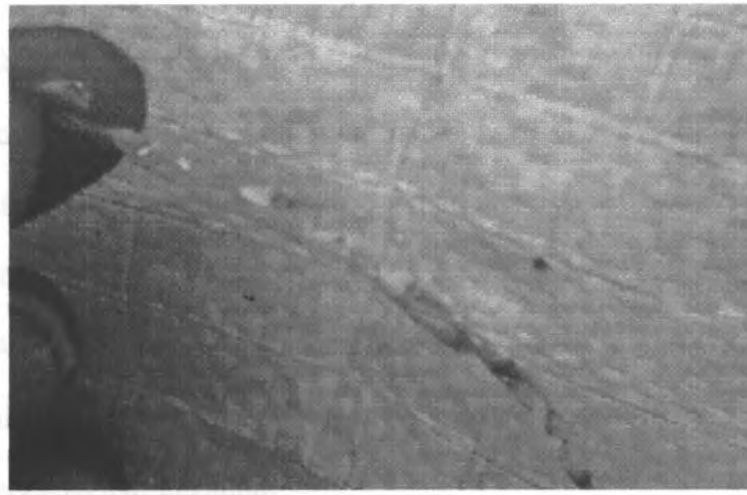

Plate 4.3: F_1 hybrid seeds formed in crosses between *O. sativa* and *O. longistaminata.*

These results indicate that hybridization between Asian rice, *O. sativa* (Basmati 370) and East African wild rice, *O. longistaminata*, is possible, although only when *O. sativa* is the maternal plant and even then with only low success (6%). The low hybridization success corresponds to the findings of Causse and Ghesquiere (1991) who reported equally low seed set of 3% and 5% in crosses between the two species. The low F_1 hybrid seed set has been attributed to deterioration of hybrid embryos about 3 days after fertilization (Sano, 1989) and low pollen fertility of only 5% in (Causse and Ghesquiere, 1991).

Seed formation occurring only with *O. longistaminata* as the pollen donor has been reported in other studies (Oka, 1956; Oka and Chang 1961; Morishima *et al.*, 1992; Song *et al.*, 2003) that found successful natural hybridization between the two species only when *O. sativa was* the recipient. Unidirectional hybridization has also been reported in crosses between and other wild rice species, and in studies of many other plant species (Arnold, 1997).

However, Noldin *et al.* (2002) demonstrated that pollen flow to *O. punctata* can occur in either direction but often from the tall *O. punctata* plants to the short O. *sativa* plants. Other studies (Oka, 1988; Morishima *et al.*, 1992) indicated that floral morphological features can affect reciprocal seed set. Their studies demonstrated that cultivated species generally have short styles and stigmas (1.5 to 4.0 mm in combined length), short anthers, limited pollen viability, and brief period between opening of florets and release of pollen (between half a minute and nine minutes). On the other hand, wild rice species differ in all of these characteristics; longer styles, stigmas and anthers, and pollen that remains viable for up to twice as long as in cultivated rice thus explaining the observed unidirectional hybridization.

Seven traits were analyzed using the Principal Component Analysis (PCA). The results (Table 4.2) indicate that six traits (panicle exersion, panicle length, flag leaf length, plant height, awn length and number of seeds) are suitable morphological markers for identifying each of the plant type.

Table 4.2: Component matrix from principal component analysis (PCA).

Morphological Traits				
	1 (51%)	2 (17%)	3 (11%)	4 (8%)
Number of spikelets per plant	0.84	-0.17	0.02	0.40
Grain length	-0.82	0.15	0.10	-0.26
Awn length	-0.79	0.10	-0.11	-0.19
Plant height	-0.71	0.38	-0.04	0.46
Flag leaf length	-0.24	-0.03	0.83	0.10
Panicle length	-0.09	0.87	-0.06	0.28
Panicle exersion (cm)	0.93	0.17	0.06	0.00

Analysis of the six morphological traits gave varied results between wild, cultivated and F_1 hybrids. Comparison of plant heights between F_1 hybrids and their parents showed significant ($F_{(2, 288)} = 34$; $P<0.05$) differences where *O. longistaminata* plants were taller (176.9 cm) than the other plant types. Plants of *O. sativa* were the shortest (116.4 cm) while those of the F_1 hybrids displayed an intermediate height (149.3.4 cm) to their parents (Fig 4.1a, Appendix 8).

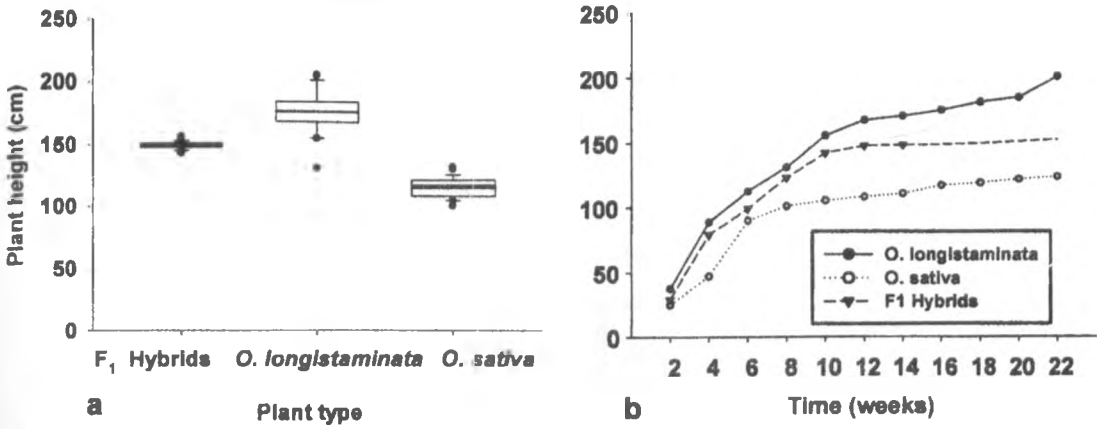

Figure 4.1: a) Mean plant height and **b)** Growth pattern in plant height in the three plant types. Mean heights were scored once at maturity but growth patterns were scored from germination time to maturity.

On the growth pattern, continued to grow in height up to maturity, but the gain in height in the hybrid and cultivar plants slowed down upon reaching the flowering stage, after the 10^{th} week (Fig. 4.1b). This may indicate that plant height could be an important characteristic for distinguish the wild rice species from either the hybrids or the cultivars. Although the growth patterns in the three plant types were similar at the early stages (2-8 weeks; the vegetative stage) of development, real differences ($P<0.05$) in height gains set in from the 10^{th} week onwards. This coincides with the stage of profuse tillering (WARDA, 1999), when rapid ground cover enables the rice crop to smother, and thus out-compete weeds. Therefore, this observation may be interpreted to mean that there was no possibility of a competitive advantage, in terms of height, of the hybrids as compared to the wild rice.

In contrast, F_1 hybrids had the longest flag leaves (43.1 cm) and *O. longistaminata* the shortest (26.2 cm; Fig. 4.2a and b; $F_{\{2, 321\}} = 29$; $P<0.05$). However, unlike in O. *sativa* and the F_1 hybrids where growth in plant decreased towards flowering

period, growth patterns of flag leaves of the three plant types continued to grow through out the entire period (4.2b).

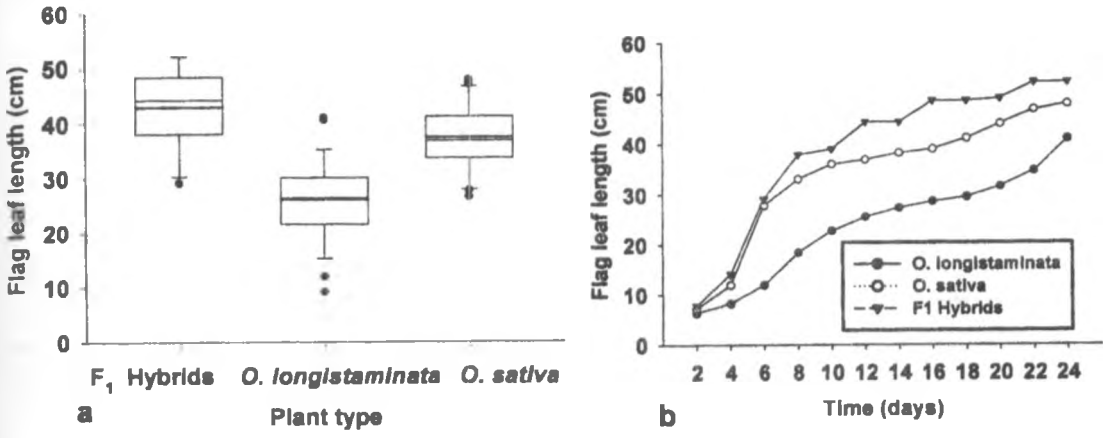

Figure 4.2: a) Mean flag leaf length and **b)** Growth pattern of flag leaf in the three plant types. Mean flag leaf lengths were scored once at maturity but growth patterns were scored every 2 days from the time of panicle initiation to the end of complete panicle formation.

A reverse in growth pattern was, however, observed in panicle exsertion; mean panicle exsertion was greatest in *O. longistaminata* (29.4 cm) while the hybrids had the least (4.6 cm; Fig. 4.3a: $F_{\{2, 288\}} = 290$; $P<0.05$). Equally, *O. longistaminata* displayed the greatest growth pattern in exsertion while the hybrids had the least (Fig. 4.3b).

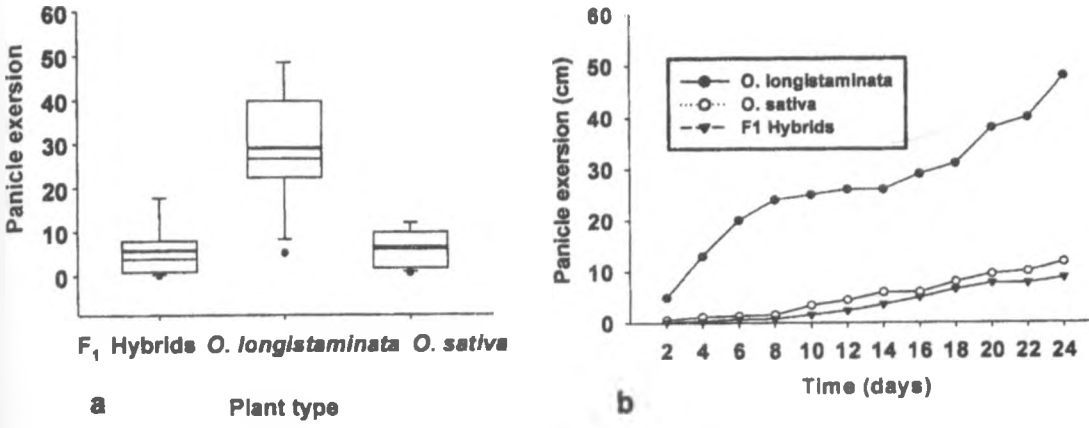

Figure 4.3: a) Mean panicle exsertion length and **b)** growth patterns in panicle exsertion in the three plant types. Mean panicle exsertion was scored once at maturity but growth patterns were scored every 2 days from the time of panicle initiation to the end of complete panicle formation.

Panicle length significantly ($F_{\{2, 417\}}=126$; $P<0.05$) varied among the plant types with the F_1 hybrids recording the highest mean length (32.1 cm) while *O. sativa* had the shortest panicle length (24.8 cm: Fig. 4.4).

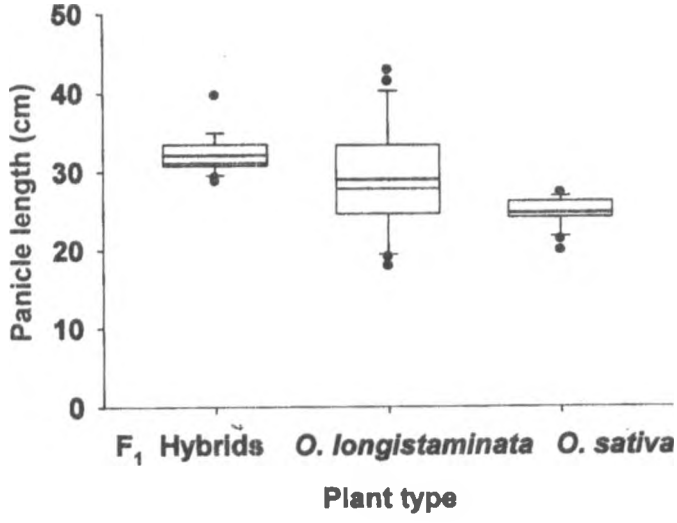

Figure 4.4: Mean panicle length; scored once at the end of complete panicle formation.

On the other hand, significant differences ($F_{\{2, 333\}}$ =258; P<0.05) were observed in awn lengths of *O. longistamianta* and those of the other two plant types. The wild plants had the longest awn length (6.4 cm) while *O. sativa* had the shortest (1.0 cm) and hybrids had an intermediate length (1.2 cm; Fig. 4.5).

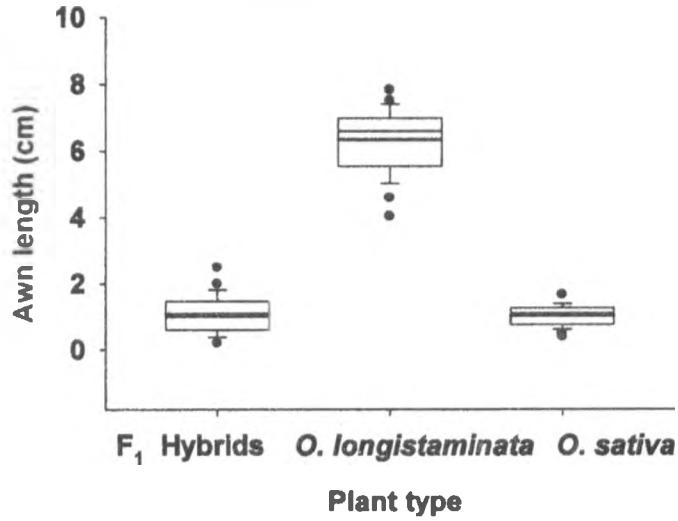

Figure 4.5: Box plot showing the mean awn length between the F_1 hybrids and parents.

The results provided conflicting information regarding plant traits. Of the six traits studied, cultivar plants displayed either an intermediate position in panicle exsertion and flag leaf length or the lowest values in panicle length and awn lengths. Inversely, the wild plants had among the highest values in plant height, panicle exsertion and awn length, an indication of an adaptive ability to the harsh wild environments (Jackson, 1985). The results point to manifestations of co-dominance in certain traits such as plant height and awn length, over-dominance in others; panicle length and flag leaf length and

under-dominance in panicle exsertion. Possession of traits that show over-dominance in hybrids is an evidence of hybrid vigour and possible heterosis.

Overall, a combination of the five traits (plant height, panicle exsertion and length, flag leaf length and awn length) indicated that hybrids were intermediate (Fig. 4.6) between the two parents and that they were closely related to the maternal (*O. sativa*) than to the paternal (*O. longistaminata*) parent.

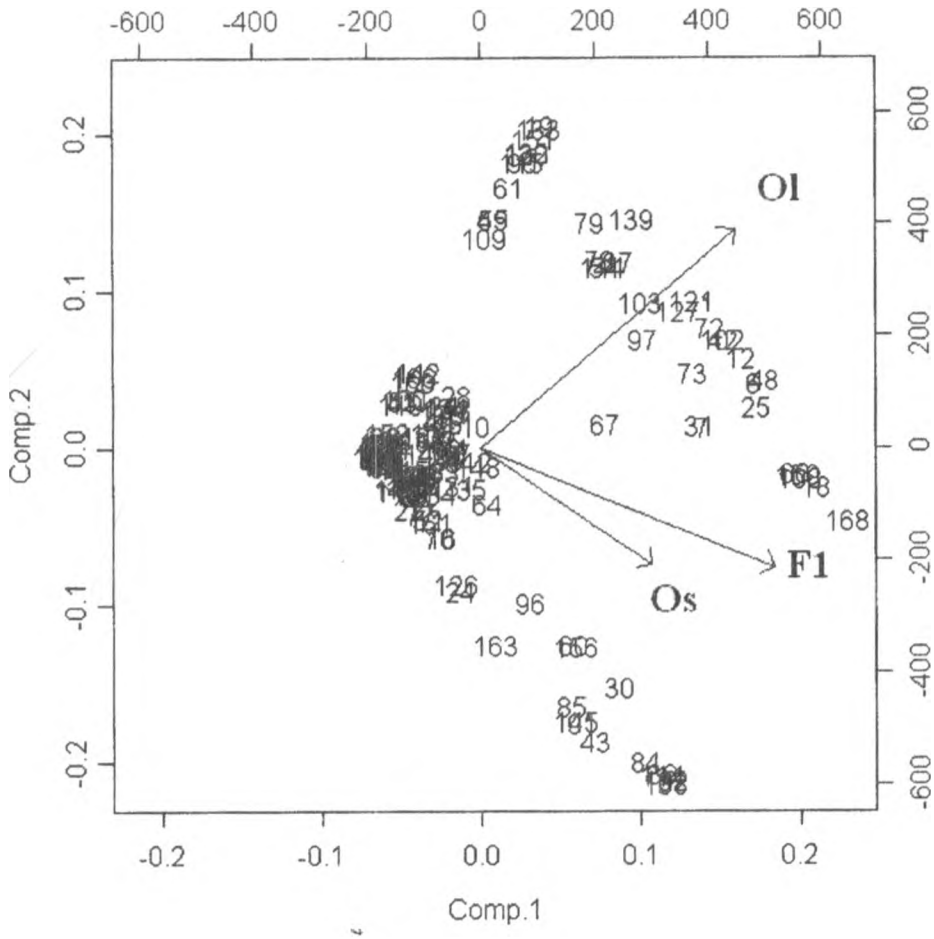

Figure 4.6: Relationship between the parents; *O. longistaminata* (Ol), *O. sativa* (Os) and the F_1 hybrids using morphological traits.

On seed production, F_1 hybrids produced the highest number of seeds per plant while the wild parents (*O. longistaminata*) produced the least (Fig. 4.6) ($F_{\{2, 501\}} = 413$; $P<0.05$).

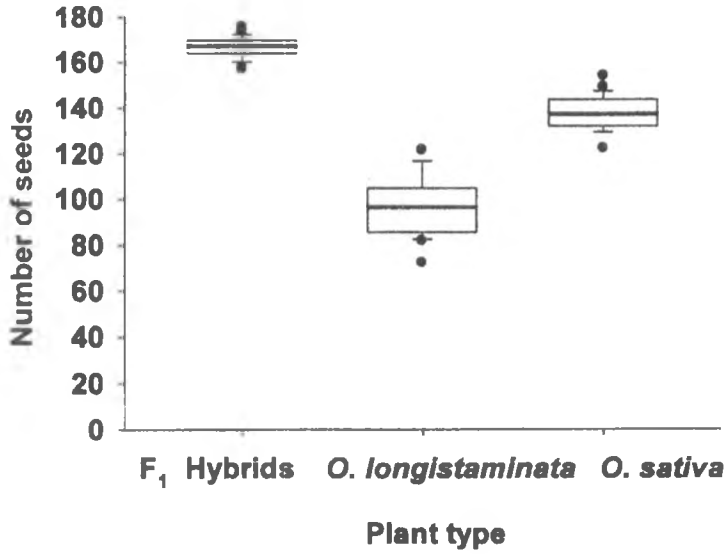

Figure 4.7: Mean number of seeds produced by F_1 hybrids and parents.

Correlation of seed production with traits among the plant types revealed mixed results. While in F_1 hybrids seed production correlated with flag leaf length ($r=+0.843$; $P<0.05$; Fig. 4.8), in *O. longistaminata* it correlated with plant height ($r=+0.767$; $P<0.05$) and panicle lenth ($r= +0.664$; $P<0.05$; Fig.4.9). In *O. sativa,* seed production correlated with plant height ($r= +0.741$; $P<0.05$) and panicle exsertion. ($r= +0.854$; $P<0.05$; Fig.4.10).

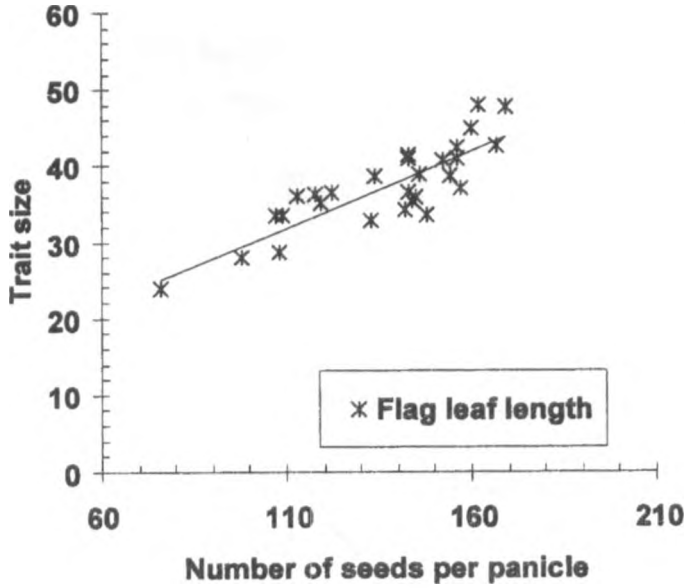

Figure 4.8: Correlation of seed production with flag leaf length in the F_1 hybrid plants.

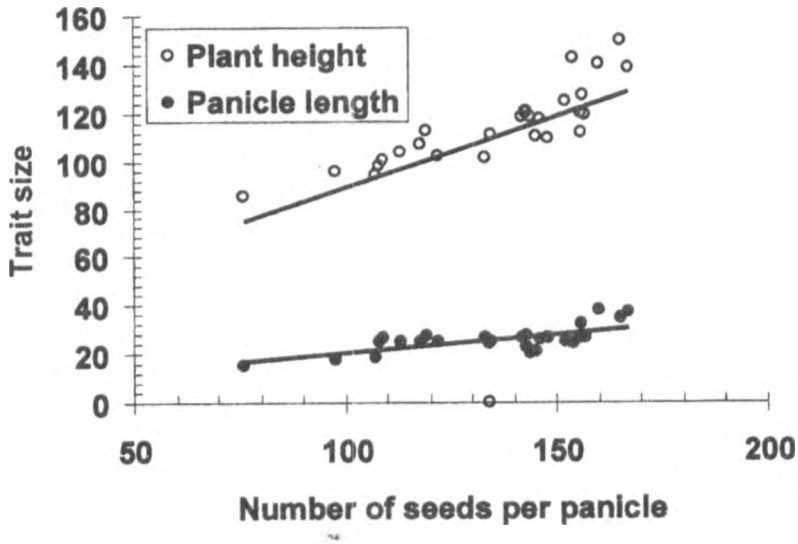

Figure 4.9: Correlation of seeds production with plant height and panicle exsertion in *O. longistaminata*.

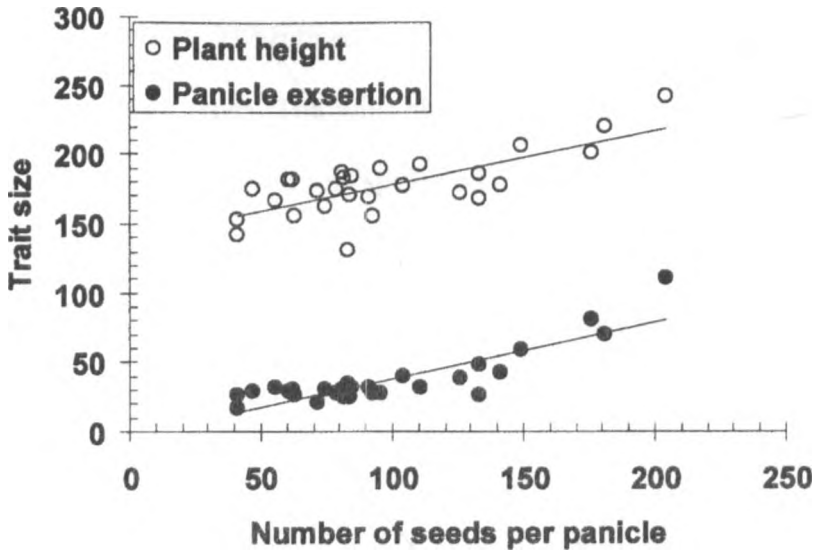

Figure 4.10: Correlation of seed production with plant height and panicle exsertion in *O. sativa*.

Findings of this study demonstrate that hybridization between the two species produced F_1 progenies with long flag leaves. These results are in line with the findings of Dere and Yildirim (2006) which established that high seed production in rice correlated with well established flag leaves. According to the study by Dere and Yildirim (2006), long and broad flag leaves attribute to increased surface area caused by increased leaf canopy hence high productivity. Recent studies (Blake *et al.*, 2007) have indicated that flag leaves, compared to other leaves, contribute most photosynthetic assimilates that are important in the grain filling. According to Dere and Yildirim (2006), flag leaves in cereals contribute about 41-43% of photosynthetic assimilates that go into grain filling and this is attributed to its length and width. A study by Watanabe and Kitagawa (2000) showed that distribution of assimilates from leaves varied depending on the position of the leaf. According to their study, about 80% of assimilate from flag leaf was translocated

to the panicle and only about 5% of it came from the fifth leaf from the flag leaf (Hasegawa *et al.*, 1996). The influence of flag leaves on seed production is also observed in the other two plant types. The wild plants (*O. longistaminata*) had the shortest flag leaves which corresponded with low seed production while the cultivar plants (*O. sativa*) had intermediate flag leaves corresponding to intermediate seed production.

In this study, plant height did not reflect ecological benefits in terms of seed production. The wild species (*O. longistaminata*), despite having an outstanding height, produced the lowest number of seeds compared to the hybrids and cultivar parents. However, this observation is not in agreement with hybridization observed in other plants other than cereals whereby height in plants was shown to improve access to light hence increased seed production (Kende *et al.* 1998).

Nevertheless, unlike in many other crops where height may be considered an ecological success, in rice it is viewed as setback in that it increases lodging (falling off of plants due to weak culms) (Jackson, 1985; Kende *et al.*, 1998) and therefore short rice cultivars are preferred especially in ecological zones with strong winds. A possible explanation for the low yield in relation to height is that high investment in plant height incurs costs linked to construction and maintenance of the stem (Falster and Westoby, 2003). Tall plants, and especially in the family Poaceae, have weak flower and seed-bearing stems which are prone to falling over (or lodging) before harvest (WARDA, 1999). Hence, low seed production in *O. longistaminata* can be viewed as an adaptive feature that reduces the sink weight and hence lodging of the tall plants.

However, generation of hybrids that are capable of producing more seeds and which are taller than the cultivar plants implies that they will be subjected to high

incidence of lodging in the rice fields which would result in reduced grain filling. This would trim-down the spread of wild-crop seed mediated gene flow. Short upright growth of rice cultivars, especially at reproductive stage, is a characteristic of *O. sativa*; which enables the plant to support heavy seed heads through maturity to harvest (WARDA, 1999).

Although awn length did not show any correlation with seed production among the three plant types in this study, in other studies it has been reported to play a major role in post-harvest events (Satoh *et al.*, 1990). According to these authors, possession of long awns is considered an adaptive defensive device that reduces predatory effects of birds and other rice feeders thus increasing their survival rate. Short awns in hybrids in this study imply that bird predation may be severe among the hybrids compared to that in parent plants. The effect of predation on rice grains is a serious problem in Kenya that negatively affects rice production (Wanjogu and Mugambi, 2001) and therefore hybrid seeds predation would reduce the potential of gene flow (through seeds).

Using the findings of this study, it is worth noting that hybrids will produce more seeds than conventional cultivars and possibly the GM rice in the event of their introduction. This suggests that seed mediated gene flow, from the hybrids, may be higher than that experienced in conventional rice. This gives a caution that unless the hybrids are weeded, cycles of resowing and high shattering may occur as high shattering is a dominant trait in *O. longistaminata* (Lin, *et al.*, 2007). Inheritance of genes responsible for shattering may allow evolution of weedy types of hybrids and is known to occur in other weedy rice types (Ferrero and Vidotto, 1998). The hybrid descendants will carry a mosaic of segments of the *O. longistaminata* and genomes that are partly

determined by selection on the segment genes, and among them possible transgenes, and partly by epistatic interactions and incompatibilities among genes in different segments.

Weeding may stop the cycle of shattering and resowing in fields, and the results show that F_1 hybrids are taller than the cultivated rice and can be recognized by experienced farmers using combination of other traits. However, in generations subsequent to the F_1 hybrids, recognition of the descendants will be more difficult (Sørensen *et al.*, 2007). If not weeded away, the hybrid descendants in fields may eventually spread their pollen to surrounding wild populations. But the unidirectional hybridization means that transgenes from GM rice would not spread to the wild rice populations as fast as when if gene flow is bidirectional.

CHAPTER FIVE

MOLECULAR CHARACTERIZATION OF *O. sativa*, *O. longistaminata* AND THEIR F_1 HYBRIDS

5.1 Introduction

Wild relatives of crop species have been used as sources of disease and pest resistance for crop improvement for decades (Tateoka, 1963). Wild *Oryza* species represent a rich, largely untapped source of genes for resistance to biotic and abiotic stresses, most notably to insect pests and diseases (Stich, 1990). Due to possession of the important genetic resources and compared with other important cereals such as wheat, alien gene transfer in rice has received tremendous attention in recent decades.

O. longistaminata shows significant diversity at the isozyme level and appears to be among the most distant species from within the Sativa group (Second, 1989). It is not clear whether the species, *O. longistaminata*, has intervened during domestication of *O. sativa* neither in the latter's diversification on the African continent since its introduction. This could be mainly attributed to its strong reproductive barrier that isolates it from all other species (Oka and Morishima, 1967). In spite of this barrier, it is believed that hybrid plants may be obtained, either by artificial crossing or, rarely, in seed sets collected from the wild plants along the borders of rice fields.

Morphological characterization of the F_1 hybrids (see Chapter 4) revealed a possible hybrid formation between the two species. However, characterization of the F_1 hybrids using morphological characters alone may not be adequate in identifying the

hybrids as it has low level of polymorphism and greatly affected by gene and environmental interaction. Various studies on rice breeding (Oka and Morishima, 1967; Ghesquiere, 1986; Kaushal and Ravi, 1998; Zhou *et al.*, 2003) suggest the use of a combination of morphology with molecular work in identifying hybrids.

Simple Sequence Repeats (SSR) also known as microsatellites are tandem arrays of short nucleotide repeats with 2-6 base pairs (bp) (Wu and Tanksley, 1993) and are widely dispersed in all eukaryotic genomes. Dinucleotide repeats $(AT/TA)_n$ and $(GA/CT)_n$ are commonly found in vascular plants. SSRs are co-dominant, highly polymorphic, abundant and randomly distributed markers in genomes. The markers can easily be amplified by PCR and are assumed to be selectively neutral (Akagi *et al.*, 1996; Roa *et al.*, 2000). Microsatellites have been used for studies of parentage, genetic diversity and gene flow (Chase *et al.*, 1997; McCouch *et al.*, 1997; Innan *et al.*, 1999; Baack and Rieseberg, 2007) among others.

Microsatellite markers have numerous advantages over the other markers (section 1.2.10) which include; use of relatively simple automated method, the monolocus nature of most markers, the display of co-dominant Mendelian inheritance, availability of high number of public SSR primer pairs and the effective cost per genotype and primer (similar to that of RAPD) (Rafalski *et al*,. 1991; Gurta *et al.*, 1999).

To develop tools for identification of hybrids and their descendants between Asian rice and wild, *O. longistaminata*, the study undertook to identify molecular differences between *O. sativa*, *O. longistaminata* and their F_1 hybrids using SSR markers.

5.2 Materials and Methods

5.2.1 Plant material

A set of 5 hybrid seeds from each of the eight different tillers (T_1-T_8) which were obtained from the previous experiment (F_1 seeds harvested from different maternal tillers; see Chapter 4) and a similar number of seeds for each of the parent plants (*O. sativa* and *O. longistaminata*) were planted in ten plastic pots (Plate 5.1) at the University of Nairobi glasshouse in July 2008. Eight pots contained F_1 hybrid seeds and 2 pots contained the parental seeds. Each set of seeds was planted in a separate pot making a total of 10 pots. After 2 weeks the young leaves from the resulting seedlings were harvested for DNA extraction.

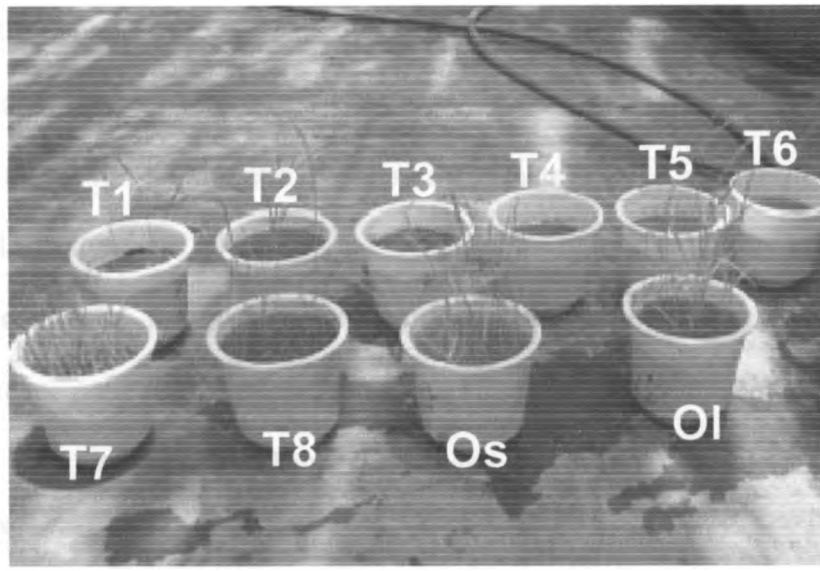

Plate 5.1: Seedlings of seeds obtained from different F_1 hybrid tillers (T1-T8) and the parents, *O. sativa* (Os) and *O. longistaminata* (Ol).

5.2.2 DNA extraction

Leaf samples obtained from one group of F_1 tillers (T1-T8) and the two parents were chopped into half-inch segments with a sterilized pair of scissors and placed in pre-chilled mortar. Liquid nitrogen was quickly added to freeze-dry the samples before grinding to fine powder. The ground material was placed in a 15ml polypropylene centrifuge tube and stored at - 20°C until used as a source of DNA.

Genomic DNA was extracted using Cetyl Trimethly Ammonium Bromide (CTAB) method described by Doyle and Doyle (1987). From the ground material, 0.3g was placed into1.5 ml eppendorf tube. Immediately, 1 ml of pre-warmed (65°C) CTAB extraction buffer (700 mM Sodium Chloride, 100 mM Tri HCL PH 7.5, 50 mM EDTA PH 8.0, 140 mM β-mercaptoethanol and 1% CTAB) was added and tubes incubated for one hour with a continuous gentle mixing in water bath (Lab-line, B7005-2) at 65°C to lyse the cells. The tubes were removed from the water bath (Lab-line, B7005-2, USA) and cooled briefly for 4-5 minutes. To the homogenate, 600 µl of chloroform/isoamyl alcohol (24:1) mixture was added and the tubes capped tightly, mixed by gentle rocking with medium circular motion using a shaker for 10 minutes. This formed a thick emulsion between the DNA phase and the chloroform/isoamyl alcohol phase. The homogenate was centrifuged (Using centrifuge-eppendorf 515R, Germany) for 10 minutes at 12,000 revolutions per minute (rpm) at room temperature (23 ± 2°C).

The top aqueous layer was pipetted out and transferred to a new eppendorf tube. To the supernatant, 600 µl of chloroform/isoamyl alcohol mixture was added, rocked gently for 10 minutes using a rotator (Lab-line, 1314R) and centrifuged at 12,000 rpm for 10 minutes at room temperature. The top aqueous layer was again pipetted out and

transferred to a new eppendorf tube and 5 µl of 10 mg/ml pre-boiled RNase A added, mixed by gentle inversion and incubated (Memmert-TV 40b incubator, Germany) for 30 minutes at 37°C.

DNA was precipitated by adding 240 µl of ice-cold isopropanol and mixed gently and left at -20°C for 5 minutes. The precipitated DNA was centrifuged at 12,000 rpm for 10 minutes (using centrifuge-eppendorf 515R, Germany) The DNA pellet was washed in absolute ethanol, centrifuged at 12,000 rpm for 10 minutes and washed again in 70% ethanol. The DNA pellet was then air-dried for 30 minutes and finally re-suspended in 100 µl of 0.1X TE (10 mM Tris, 1 mM EDTA- pH 8.0) at 37°C for 1 hour while mixing every 15 minutes by gentle inversion to help speed up the process.

5.2.3 DNA Quality and Quantity

The quality of genomic DNA obtained was assessed by agarose gel electrophoresis. A 0.8% agarose gel was prepared by weighing low melting 0.8 g of agarose in a 250 ml beaker containing 100 ml of 1X TAE buffer (0.04 M Tris-acetate and 0.01 M EDTA) and swirled to mix. The mixture was boiled in a microwave oven (GE, JES2251SJSS, USA) and allowed to cool to about 45°C. The molten gel was stained by adding 2 µl of ethidium bromide (10mg/ml ultra PURE from GIBCO BRL) before pouring it into a medium gel mold with two 11 well combs. The gel was allowed to solidify before placing it in a gel electrophoresis tank (Scie-Plas) containing 1XTBE buffer. The gel was completely submerged prior to removing the combs.

Each DNA sample was mixed with loading dye (40% (W/V) sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol) at a ratio of 5 µl of DNA to 1 µl of the dye. The

DNA mixture was then loaded into the wells, gel tank (Sie-Plas) electrodes hooked up and power (Sigma PS 2000-2 power supply) turned on to 80 volts. After running for about one hour, the gel was removed, visualized on a UV transilluminator box (Multi doc-it imagining system) and photographed using Polaroid camera and film. From the photographs, the DNA quality was checked and the sheared DNA samples were re-extracted. Traces of RNA were removed by re-incubating the DNA with 5 µl of RNAse A (10mg/ml) for 30 minutes at 37°C.

Concentration of the genomic DNA was determined on the basis of optical density (OD) readings. From each stock of DNA sample, a 2 µl aliquot was diluted to 100 µl using 1 XTE buffer, and its OD determined at wavelengths 260 and 280 nm on a spectrophotometer (DU 530 Beckman Coulter). DNA concentration in each sample was determined using the formula shown below.

DNA concentration (µg/µl) = OD260 X 50 (dilution factor) X 50 µg/ml/1000

5.2.4 Polymerase chain reaction (PCR)

After calculating the concentrations in µg/µl, the stock DNA solution was diluted to 25 ng/µl working solution for PCR using the formula;

$M_1V_1=M_2V2$

Where M1 is the stock DNA concentration

V1 is the volume of the stock to be diluted

M2 is the concentration of working solution (25 ng/µl)

V2 is the volume of working solution to be prepared (100*µl*)

The working solution was stored at -20°C.

5.5.5. Amplification (SSR analysis)

A total of 22 SSR primers (Appendix 9) obtained from Inventrogen Corporation, U.S.A, were screened and 6 primers (Table 5.1) were selected based on their high polymorphism information content (PIC) (Cho *et al.*, 2000).

Table 5.1: List of polymorphic rice SSR markers obtained from Inventrogen Corporation, U.S.A; Tm is the melting temperature for the primer and 'Repeat' represents the number of times the sequence is repeated (Cho *et al.*, 2000; http://www.gramene.org)

Primer ID	Forward 5'-3'	Reverse 5'-3'	Fragment size	Tm (°C)	Repeat
RM44	ACGGGCAATCCGAACA ACC	TCGGGAAAACCTACCC TACC	271	56	(GA)16
RM167	GGAAGGTAACTGTTTC CAAC	GAAATGCTTCCCACAT GTCT	128	55	(GA)16
RM180	CTACATCGGCTTAGGT GTAGCAACACG	ACTTGCTCTACTTGTG GTGAGGGACTG	85	55	(ATT)10
RM234	ACAGTATCCAAGGCCC TGG	CACGTGAGACAAAGA CGGAG	156	55	(CT)25
RM263	CCCAGGCTAGCTCATG AACC	GCTACGTTTGAGCTAC CACG	200	57	(CT)34
RM280	GATCCAGCGTGAAGGA ACACGT	AGTCCGACCACAAGGT GCGTTGTC	156	55	(GA)16

PCR was performed in 10 µl reaction mixes consisting of 25 ng template DNA, 1.5 mM $MgCl_2$, 0.8 mM dNTP mix, 0.5 µM SSR primers (forward and reverse), 0.125 U Taq

polymerase (Roche) and 1 X PCR reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$). In a 200 µl PCR tube, 9 µl of master mix was mixed with 1 µl of 25 ng/µl DNA and run in a PTC100 thermocycler (USA) with the following PCR program; Initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 55°C for 45 seconds and 72°C for 30 seconds. This was followed by one final extension cycle at 72°C for 10 minutes, and an indefinite hold at 4°C. The SSR amplification products were resolved on 2% agarose gel in 1X TAE buffer. Gels were run in a medium format horizontal gel system at 100V for 45 minutes and were photographed under UV light.

5.2.6 Genotyping

The six selected markers were used for fluorescent genotyping. The forward primer for each of the markers was labelled at the 5' end of the oligonucleotide with one of the following fluorescent dyes: 6-FAM (blue), PET (Red), VIC (Green) and NED (Yellow) (Applied Biosystems), allowing post-PCR pooling of the 6 primer products into two groups of three primer products each (Table 5.2).

Table 5.2: Co-loading sets1 and 2; bp represents size of the base pair and 'proportion' represents the proportion of PCR products.

Set 1

SSR's	Colour	Size (bp)	Proportion
RM44	YELLOW	128	0.7ul
RM167	RED	128	1.1ul
RM180	BLUE	85	0.5ul

Set 2

SSR's	Colour	Size (bp)	Proportion
RM234	YELLOW	156	1.0ul
RM263	RED	200	1.0ul
RM280	GREEN	156	1.6ul

All PCR reactions were performed in 96 well plates in a thermocycler using the previously optimized PCR conditions. All the 10 samples for every primer pair were amplified and tested on 2% agarose gels before the PCR products were used in fragment analysis. One microliter (1.0 µl) each of the PCR products were pooled and 7 µl cocktail mixed (HIDI formamide, with 12 µl of 500 size standard) were added. The mixture was denatured at 95°C for 3 minutes, quickly chilled in ice and then loaded in an applied biosystems (ABI) PRISM 3730 capillary electrophoresis sequencer. PCR products were pooled together and co-loaded in proportions shown in Table 5.1. Fragments analysis was done using the genemapper software version 3.7 after each run.

5.2.6 Statistical analysis

Statistical data analysis was performed using PowerMarker version. 2.5 (Liu and Muse, 2005). Polymorphic Information Content (PIC) for each primer was determined as described by Weir (1996):

$$He=1-\Sigma Pi^2$$

Where, Pi is the frequency of the i^{th} allele.

Genetic diversity was measured in terms of number of alleles per locus using Nei's unbiased estimate of gene diversity (He) (Nei, 1987) (Table 5.5). The differences in value between expected (He) and observed heterozygosity (Ho) provided an indication of deviations from random mating in relation to Hardy-Weinberg (H-W) equilibrium (Distance matrices were calculated on the basis of Euclidean distance (Wright, 1978).

In order to quantify the genetic relationship or relatedness of the hybrids and the parents, cluster analysis was done based on distance matrices by using the unweighted pair group method analysis (UPGMA) based on Euclidean genetic distances (Nei, 1987). The relationships between parents and the hybrids were represented graphically in the form of dendrogram.

5.3 Results and Discussion

5.3.1 DNA analysis

The genomic DNA bands (Fig.5.1) were stable, free from the RNA and equally of good quality since there was no shearing. Electrophoretic agarose gel analysis revealed uniform mobility and the absence of residual salts and other impurities in the wells of the quantification gel was an indication of high quality DNA. Agarose gel electrophoresis is

the most appropriate technology for routine check up and it is more widely available gel system (Cho *et al.*, 2000). However, use of agarose electrophoresis does not reveal allelic variation since PCR products differing by few (e.g. two) base pairs cannot be resolved with agarose gel (Cho *et al.*, 2000).

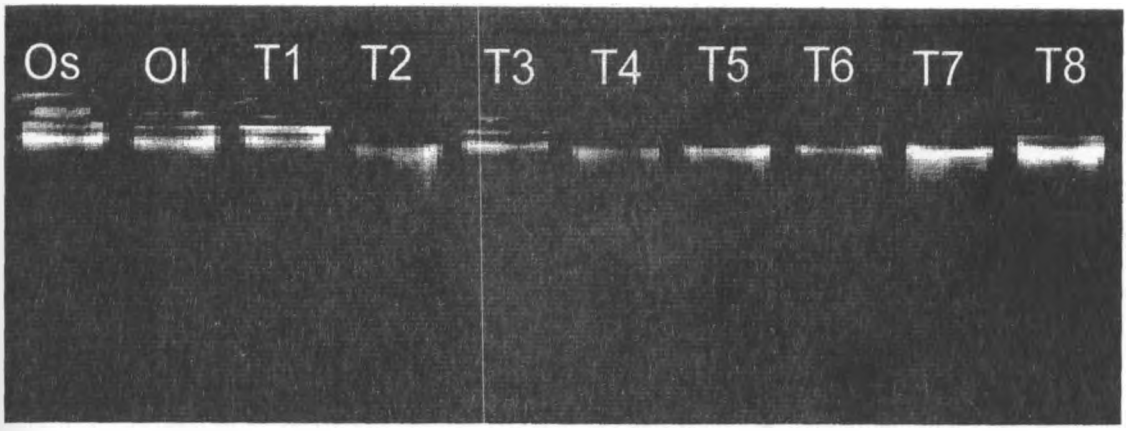

Figure 5.1: Genomic DNA resolved with 0.8% agarose; the DNA was extracted from F_1 hybrid tillers (T1-T8) and parent plants; *O. sativa* (Os) and *O. longistaminata* (Ol).

5.3.2 DNA purification and quantification

The DNA quality was checked for contaminants using spectrophotometer (DU 530 Beckman Coulter) readings at wavelengths of 260 and 280 nm. The ratio of the optical densities ranged between 1,833 and 2,001 (Table 5.3). This was indicative of relatively pure DNA, not contaminated with proteins, RNA, and carryover chemicals from extraction buffers and reagents. After re-precipitation of the contaminated DNA, all the 10 DNA samples studied had an OD ratio ranging from 1.8 to 2.0, which is the standard required DNA purity (Doyle and Doyle, 1987). The concentration of genomic DNA ranged from 95-740µg/ml with a mean of 416.5 µg/ml. This indicates that the CTAB method of DNA extraction was reliable and can be applied from one lab to the other.

Table 5.3: Specrophotometric measurements of A260/A280 ratio and DNA concentration *O. sativa* (Os), *O. longistamianata* (Ol) and F_1 hybrids (T1-T8).

Sample	260nm	280nm	260/280	DNA conc. (ng/µl
Os	0.412	0.214	1.925	95
Ol	0.146	0.077	1.896	365
T1	0.077	0.042	1.833	192.5
T2	0.131	0.068	1.926	327.5
T3	0.284	0.142	2.000	710
T4	0.296	0.155	1.910	740
T5	0.114	0.059	1.932	285
T6	0.039	0.021	1.857	97.5
T7	0.279	0.139	2.001	697.5
T8	0.261	0.139	1.878	652.5

5.3.3 SSR analysis

Results of the Applied Biosystems (PRISM 3730 capillary electrophoresis sequencer) DNA analyzer showed that the six microsatellite markers detected a total of 12 alleles in the entire data set (Table 5.4). The number of alleles per primer was two and all the six markers were found to be polymorphic.

Table 5.4: A summary table showing size of various alleles detected in the hybrids (T1, T3, T5, T6 and T7; bolded yellow) and parents; *O. sativa* (Os) and *O. longistaminata* (Ol).

Marker	Sample Name									
	Os	Ol	T1	T2	T3	T4	T5	T6	T7	T8
RM180	157	101	157	157	**101**	157	157	**101**	157	157
RM180	157	101	157	157	**157**	157	157	**157**	157	157
RM234	124	126	124	124	124	124	**124**	124	124	124
RM234	124	126	124	124	124	124	**126**	124	124	124
RM263	158	156	158	158	**156**	158	158	158	**156**	158
RM263	158	156	158	158	**158**	158	158	158	**158**	158
RM280	166	168	168	166	168	166	166	166	**166**	166
Rm280	166	168	168	166	168	166	166	166	**168**	166
RM44	111	103	**103**	111	111	111	111	**103**	111	111
RM44	111	103	**111**	111	111	111	111	**111**	111	111
RM167	128	140	**128**	128	128	128	128	128	128	128
RM167	128	140	**140**	128	128	128	128	128	140	128

The markers were able to distinguish parental genotypes by detecting either one or two alleles in the hybrids. RM180 detected alleles of sizes 101 and 157 base pairs in the parents and two hybrids (T3 and T6). RM234 detected two alleles of sizes 124 and 126 base pairs and in the parents and one hybrid (T5). RM263 detected alleles of sizes 156 and 158 base pairs in the parents and in hybrids T3 and T7 while RM280 identified alleles of sizes 166 and 168 base pairs in the parents and in hybrid T7. Alleles of sizes 103 and 111 base pairs were detected by marker RM44 in parents and hybrids (T1 and T6) while RM167 identified alleles of sizes 128 and 140 in parents and hybrids (T1 and T7).

Alleles were represented in form of electropherograms (Fig. 5.2 and 5.3) that showed the peaks at which each allele was detected.

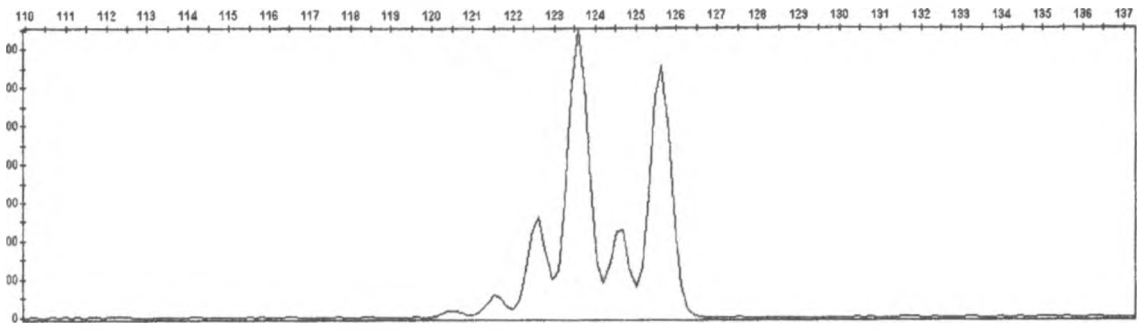

Figure 5.2: Electopherogram showing alleles of sizes 103 and 111 base pairs; Amplification used DNA marker RM234 and the DNA samples were obtained from hybrid tiller six (T6).

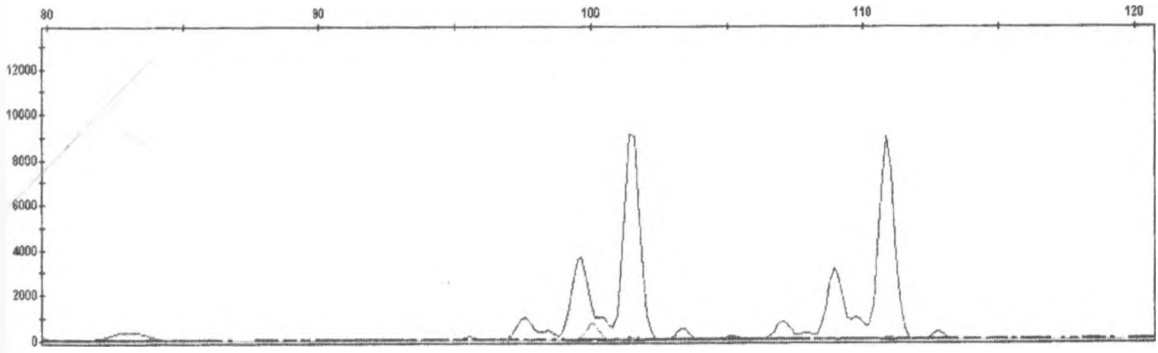

Figure 5.3: Electopherogram showing alleles of sizes 124 and 126 bp; Amplification used DNA marker RM44 and the DNA samples were obtained from hybrid tiller one (T1).

The electropherograms clearly showed that the screened markers were polymorphic and were able to distinguish hybrids from the parents. However, some of the plants (T2, T4 and T8) which had been previously characterized as hybrids (during morphological characterization) were found to be genetically similar to the maternal plant and were therefore disqualified from being regarded as hybrids (Fig. 5.4). This indicates that

although pollination in certain parental tillers seemed to have been successfully performed, no effective hybridization occurred. The results revealed that either not all of the maternal (*O. sativa*) flowers received pollen from the paternal plants (*O. longistaminata*). Alternatively, the paternal pollen (foreign) may have been out competed for fertilization by the 'maternal' pollen grains resulting in self pollination. It is also possible that there was human error during emasculation that resulted into selfing. According to Bannert and Stamp (2007) foreign pollen is mostly outcompeted by the maternal's pollen due to their high vigour and density. These results therefore show that morphological traits are relatively less reliable and efficient for precise discrimination of closely related plants and analysis of their genetic relationship.

Equally, the PIC values varied across the markers with RM234 recording the lowest 0.223 while RM280 recorded the highest (0.354; Table 5.5). PIC values give the information on the impact of each marker, which is the measure of the usefulness of each marker in distinguishing one individual from another. PIC is influenced by the number and frequency of alleles and it is a valuable indicator of a marker polymorphism (Liu *et al.*, 2000). A high PIC value indicates that the marker is highly informative and can distinguish closely related genotypes.

In the present study the PIC values and diversity were relatively lower (the highest recording less than 50%) than those previously reported in rice (>50%; Cho *et al.*, 2000). The explanation for this could be attributed to the maximum number (2.0) of different alleles expected in the working samples (one from each parent). The influence of allele frequency on the PIC value was explicit, a suggestion that hybridization between the two species occurred as exemplified by the presence of alleles from each parent in the

hybrids. Markers with high heterozygosity (RM280 and RM263; Table 5.5) showed high PIC values indicating that some markers are more useful than others for differentiating closely related tillers.

Based on mean number of alleles and Nei's unbiased estimate of gene diversity, RM280 and RM263 showed the highest (0.459 and 0.375 respectively) diversity within the parents and among the hybrids. However, Nei's unbiased estimates of mean number of alleles were very low (Table 5.5) within and among the parents and hybrids. This is an indication of limited variation within the hybrids caused by the low levels of heterozygosity. The low level of heterozygosity (ranging from 0.1 to 0.4) across the loci could be explained by the non-random mating nature (as they were controlled crosses) of the species under study. However, the allele's frequency had mixed results ranging from 0.15 for alleles of size 128 bp to 0.80 for the alleles of size 135 bp (Table 5.5).

Table 5.5: A summary table showing number of alleles, gene diversity, heterozygosity and the PIC values.

SSR Marker	Number of alleles	Bp of observed alleles	GeneDiversity	Frequency	Heterozygosity	PIC	Variance	SD
RM44	2.000	111,107	0.320	0.200	0.200	0.269	0.011	0.105
RM167	2.000	140,128	0.320	0.800	0.200	0.269	0.011	0.105
RM180	2.000	101,157	0.320	0.800	0.200	0.269	0.011	0.105
RM234	2.000	128,135	0.355	0.357	0.300	0.323	0.017	0.132
RM263	2.000	156,158	0.375	0.250	0.350	0.305	0.016	0.125
RM280	2.000	166,170	0.459	0.150	0.429	0.354	0.010	0.101
Mean	2.000		0.342	0.200	0.230	0.281	0.011	0.105

5.3.4 Genetic relationship

Comparison of genetic relationship among the parents and hybrids revealed a clear separation of parents from the hybrids (Fig. 5.4). By means of a dendrogram, the ten samples were assembled into clusters in different levels (called groups). The first group consisted of *O. longistaminata* while group ii consisted of *O. sativa* and the F_1 hybrids. Group iii consisted of hybrids (T3 and T6) but group iv gave two other clusters (cluster v and vi). Cluster v consisted of two hybrids (T1 and T7) while cluster vi consisted of group vii (hybrids T5) and a further cluster viii of T2, T4 and T8 (which were previously identified as hybrids by morphological markers) and the maternal parent (Os).

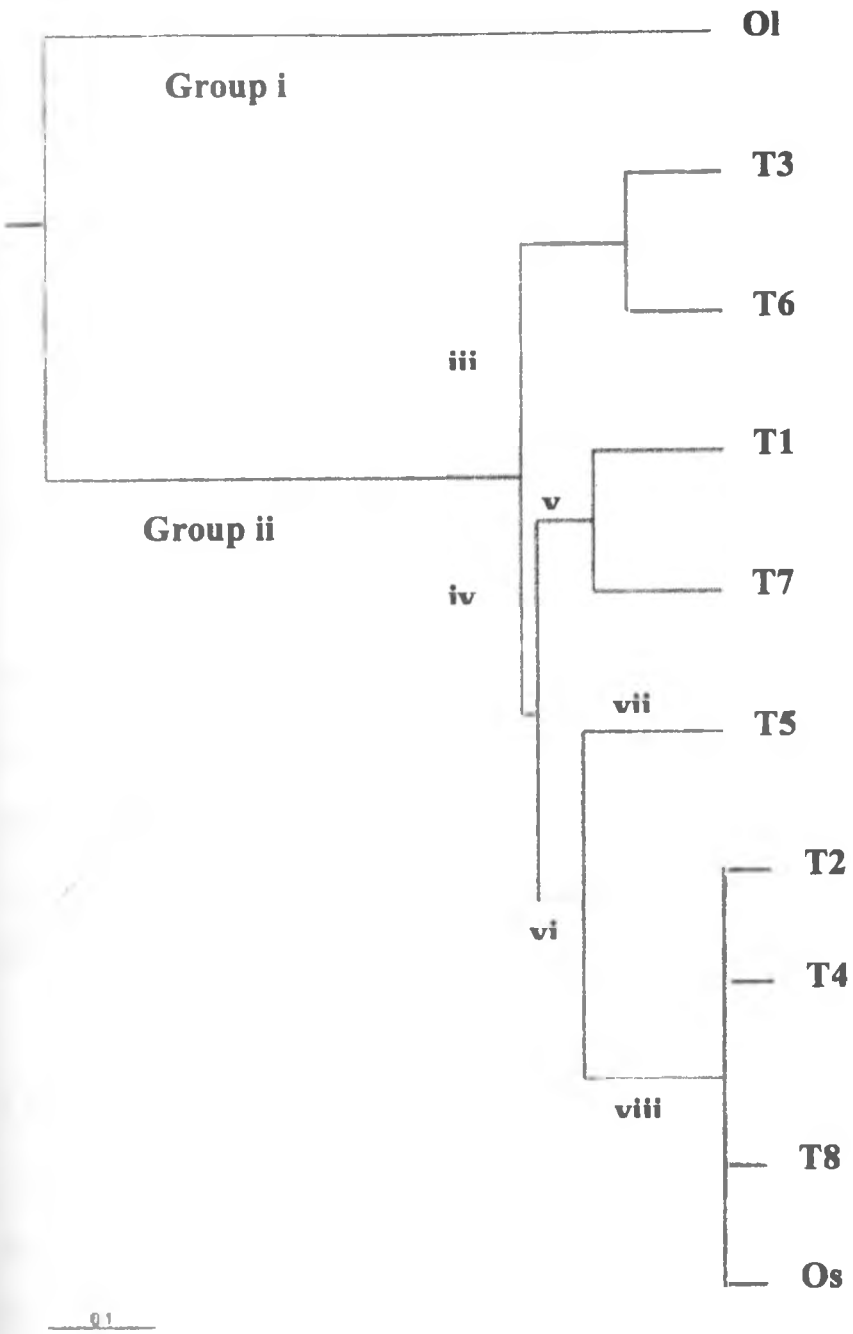

Figure 5.4: Dendrogram generated for the eight F_1 (T1-T8) and two parents *O. sativa* (Os) and *O. longistamianta* (Ol) using six SSR markers based on Nei's (1987) genetic distance.

The present study also suggests that despite the covering of emasculated flowers with porous papers to prevent any self pollination, there is a possibility that in some tillers maternal pollen made their way in large numbers into the emasculated flowers thus causing self fertilization. This further confirms the morphological characterization results (Chapter 4) which showed that certain tillers which were considered as hybrids displayed certain morphological traits (such as flag leaf length) similar to those of *O. sativa*. Thus molecular analysis isolated the true hybrids from plants (T2, T4 and T8) that had been 'falsely' identified by morphological markers as hybrids.

The genetic relationship (Fig. 5.4) revealed pure hybrid plants from their parents although the analysis failed to show a clear relationship among the hybrids and the paternal parent (*O. longistaminata*) as there was minimal clustering between the hybrids and the wild rice. However, formation of hybrids (T3, T6, T1, T7 and T5) was a clear indication that hybridization did occurr. This was shown by clustering of the hybrids further from the two parents.

5.3.5 Genetic distance

Using the Nei's (1987) identities and distances, cluster i (Ol) and cluster viii (T2, T4, T8 and Os) had the highest genetic distance of 1.414 while the lowest (0.000) was between the members of cluster Viii (T2 and T4) (Table 5.6).

Table 5.6: Genetic distances among the hybrids (T1-T8) and the parents; *O. sativa* (Os) and *O. longistamianata* (Ol).

Output	T1	T2	T3	T4	T5	T6	T7	T8	Ol	O
T1	0.000									
T2	0.282	0.000								
T3	0.565	0.282	0.000							
T4	0.353	0.000	0.282	0.000						
T5	0.424	0.176	0.353	0.282	0.000					
T6	0.353	0.353	0.176	0.353	0.530	0.000				
T7	0.235	0.282	0.282	0.353	0.282	0.530	0.000			
T8	0.353	0.000	0.282	0.000	0.282	0.353	0.353	0.000		
Ol	1.060	1.141	1.131	1.414	1.131	1.060	1.060	1.414	0.000	
Os	0.353	0.00	0.282	0.000	0.282	0.353	0.353	0.000	0.414	0.

Genetic distance is a measure of the dissimilarity of genetic material between different species or individuals of the same species (Nei, 1987). By comparing the percentage difference between the same genes of different species, a figure is obtained, which is a measure of "genetic distance". Depending upon the difference, and correcting this for known rates of evolution, genetic distance is used as a tool to construct dendrogram showing the relatedness among species or individuals. In the present study, the two species appear to be distantly related than their hybrids.

CHAPTER SIX

GENERAL CONCLUSION AND RECOMMENDATION

6.1 Conclusion

From the present study it can be deduced that for effective germination of *O. longistaminata* seeds a combination of physical (seed dehulling) and chemical dormancy breaking methods should be applied. However, seed dehulling is the only single factor that should be applied before other treatment methods are employed as this is critical for enhanced germination of *O. longistaminata* seeds. The present study observed that application of individual treatments rather than a combination of them has a greater positive effect on germination. Generally, for effective germination of seeds, the seeds should first be dehulled and then followed by germination in GA at high temperatures (31°C). Further, the study concludes that incubation of *O. longistamianta* seeds has minimal positive effect on germination.

The study also found that an isolation distance of more than 250 m between fields of GM rice and other rice fields including wild *Oryza* populations may minimize gene flow. This may however, not eliminate the probability of pollen flow from GM fields, as extreme conditions promoting pollen flow at longer distances may occur irregularly. However, this will depend on various climatic factors such as wind dynamics and temperature regimes since extreme conditions promoting pollen flow at longer distances may occur irregularly. The study also demonstrated that movement of pollen grains at different heights was influenced by wind dynamics.

Despite the findings of this study indicating no significant difference in pollen dispersal between the two heights, there is strong evidence that increased height range between the two heights would result in differences in pollen dispersal where more pollen grains could be captured at lower heights near the pollen source than the greater heights at the same distance and the vice versa for further distances away form the pollen source. The results of vertical rice pollen movement can serve as a guide for selection of an effective buffer crop to be planted between the transgenic rice and the wild rice when there is insufficient space between the former and the latter. The study also indicated that time of the day is crucial for pollen dissemination. This information on temporal rice pollen production would help in the management of GM rice pollen in that farmers would be advised of the appropriate time to take care of the large pollen density. Knowledge on pollen density and wind direction would help farmers in farm arrangements given that strong winds mostly blow in the afternoons. Farmers would be advised to plant GE rice crop on the orientation to which wind is blowing so as to reduce pollination of cultivated rice by the GE rice.

The present study confirmed that hybridization between *O. sativa and O. longistaminata* can occur which is a strong indication that the wild species would also hybridize with the GE rice. However, the risks associated with the introduction of GE rice may be minimal given that gene flow would be expected to occur mainly from the wild rice to the cultivated rice and not the vice versa. The unidirectional gene flow also suggests that most hybrids will initially be found in the cultivated fields. The F_1 seeds will be harvested, and if the farmer uses own seeds for sowing next crop, as is common in East Africa and other regions of the world with small scale cultivation, the F_1 hybrids

may grow, reproduce, and shatter their seeds within fields. Over generations, this may result in inbreeding among the hybrids and finally segregation of the parent genes if there is no introgression. This would mean that even if gene flow occurs between the two species, there would be no ecological consequence due to lack of fitness of hybrids.

The study also showed a great morphological variability among the hybrids and parents and it is therefore imperative that morphological traits be used as markers for identification of the hybrids. In particular, F_1 hybrids can be distinguished from their parents by use of panicle and flag leaf lengths. In addition, the hybrids produced more seeds than either of the parents an indication of hybrid vigour. This gives a strong signal that seed mediated gene flow may be higher in the hybrids formed from the GE rice than that experienced in conventional rice. Using information from the present study it can be generally concluded that despite lack of precise discrimination of closely related species, morphological markers are useful for preliminary evaluation as they are fast, simple, and cheap and can be used as a general approach for assessing genetic diversity among morphologically distinguishable plant types.

The molecular study confirmed that hybridization between the two species occurred. However, the molecular markers were more specific and accurate than morphological markers. Compared to other studies of SSR diversity in rice (Cho *et al.*, 2000), this study revealed 2.0 averages of alleles in the 6 loci studied. There was a high degree of average relatedness among 9 plant types (hybrids and the maternal parent) with the *O. longistaminata* showing a separate genome variation and probable structure. The minimum genetic distance from the study confirmed the ability of SSR markers to distinguish closely related genomes. It can also be concluded that the SSR markers were

informative in the germplasm studied with RM280 being the most informative marker as exemplified by the PIC value. High PIC values indicate that the marker could differentiate individuals with a difference of only two base pairs.

The number and frequency of alleles at different DNA marker loci found in this study had not been previously reported in crosses between the two species. This has allowed for analysis of the hybrids formed between a cultivated rice plant and a wild rice plant at the molecular level from East Africa. The present study thus provided reference data with representative alleles across the 6 loci, to allow comparison with future diversity assessments. The study also provided protocols for any future genotyping work involving the 6 SSR's in rice studies especially the wild type of East Africa. Since the mean number of alleles and Nei's unbiased estimate of gene diversity (He) are indicators of genetic diversity, this study revealed that there was but limited genetic diversity between the cultivated rice and the wild rice.

6.2 Recommendations

Based on results of this study, the following are suggestions for management actions that may mitigate gene flow from the GM rice to the cultivated and the wild rices.

- Further work on reciprocal hybridization of the two species in different environments especially in habitats where *O. longistaminata* naturally grows is recommended.
- Stability and fitness of the hybrids should be checked over time with a view of establishing how they can withstand the various agronomic stresses.

- For synchronization of the flowering time and subsequent hybridization between the two species, *O. longistaminata* should be planted 3 months earlier when using the vegetative plant material. However, when using seeds the wild species should be planted 3 weeks earlier.

6.3 Suggested further research

There still remain a number of aspects of gene flow between the two species that are not well understood. The following have been identified as priority areas for research in the context of environmental risk assessment.

1). Natural hybridization between the two species needs to be assessed to synchronize the results of artificial hybridization. In this, emphasis should be directed to: -

   (i). the settling and deposition rate of the rice pollen on the receptor flower

   (ii). Viability of the pollen grains at various distances along a specific wind speed

   (iii). Rate of hybridization at various distances

2). Assessment of stability and fitness of the hybrids from F_1 to F_6 generations.

   The following should be investigated:-

   (i). Detection of the genes responsible for resistance to various diseases and other traits.

   (ii). Nutritional content of the F_1 hybrids.

3). Population studies with the SSR markers tested in this study should be conducted to determine to what extent hybridization really takes place in and around typical East African rice fields.

4). The study recommends for extensive studies on morphological markers 1) in different regions and agro-ecological zones, 2) on various edaphic factors and topographies to determine whether other than the genetic factors extraneous factors such as nitrogen and phosphorus levels influence the hybrid vigour. This is so especially in East Africa where topography and temperature regimes greatly influence climatic conditions of the region.

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APPENDICES

Appendix 1: ANOVA table displaying the effects of germination by temperature, incubation, chemical media and their interaction. Inc (incubation), Temp (Temperature) and * (interaction).

Dependent Variable: Percent

Source of variation	df	Sum of Squares	Mean Square	F	Sig.
Inc	1	23166.964	23166.964	48.6	0.000***
Temp	1	5916.964	5916.964	12.413	0.000***
Medium	2	68802.844	34401.422	72.167	0.000***
Inc * Temp	1	7.44	7.44	0.016	0.901
Inc * Medium	2	441.071	220.536	0.463	0.630
Temp * Medium	2	301.389	150.694	0.316	0.729
Inc * Temp * Medium	2	750.198	375.099	0.787	0.456
Error	324	154448.016	476.691		
Total	335	253834.888			

Appendix 2: a) Emasculation and **b)** Emasculated flowers covered with porous papers to prevent any further external pollination

a b

Appendix 3: ANOVA table on plant heights of *O. sativa*, *O. longistaminata* and the F_1 hybrids

(I) sppname	(J) sppname	Mean Difference (I-J)	Std. Error	P value	95% Confidence Interval for Difference	
					Lower Bound	Upper Bound
F_1 Hybrids	*O. longistaminata*	-20.420(*)	5.771	0.000	-31.779	-9.062
	O. sativa	27.530(*)	5.786	0.000	16.142	38.918
O. longistaminata	F_1 Hybrids	20.420(*)	5.771	0.000	9.062	31.779
	O. sativa	47.950(*)	5.801	0.000	36.533	59.367
O. sativa	F_1 Hybrids	-27.530(*)	5.786	0.000	-38.918	-16.142
	O. longistaminata	-47.950(*)	5.801	0.000	-59.367	-36.533

Appendix 4: ANOVA table on flag leaf length of *O. sativa, O. longistaminata* and the F_1 hybrids

(I) sppname	(J) sppname	Mean Difference (I-J)	Std. Error	P value	95% Confidence Interval for Difference	
					Lower Bound	Upper Bound
F_1 Hybrids	*O. longistaminata*	12.996(*)	1.822	0.000	9.412	16.58
	O. sativa	3.097	1.856	0.096	-0.555	6.749
O. longistaminata	F_1 Hybrids	-12.996(*)	1.822	0.000	-16.58	-9.412
	O. sativa	-9.899(*)	1.78	0.000	-13.401	-6.397
O. sativa	F_1 Hybrids	-3.097	1.856	0.096	-6.749	0.555
	O. longistaminata	9.899(*)	1.78	0.000	6.397	13.401

Appendix 5: ANOVA table on panicle exsertion of *O. sativa, O. longistaminata* and the F_1 hybrids

Sppname	(J) sppname	Mean Difference (I-J)	Std. Error	P value	95% Confidence Interval for Difference(a)	
	panicle				Lower Bound	Upper Bound
F_1 Hybrids	*O. longistaminata*	-22.688(*)	0.64	0.000	-23.948	-21.428
	O. sativa	-0.946	0.64	0.140	-2.206	0.314
O. longistaminata	F_1 Hybrids	22.688(*)	0.64	0.000	21.428	23.948
	O. sativa	21.742(*)	0.64	0.000	20.482	23.002
O. sativa	F_1 Hybrids	0.946	0.64	0.140	-0.314	2.206
	O. longistaminata	-21.742(*)	0.64	0.000	-23.002	-20.482

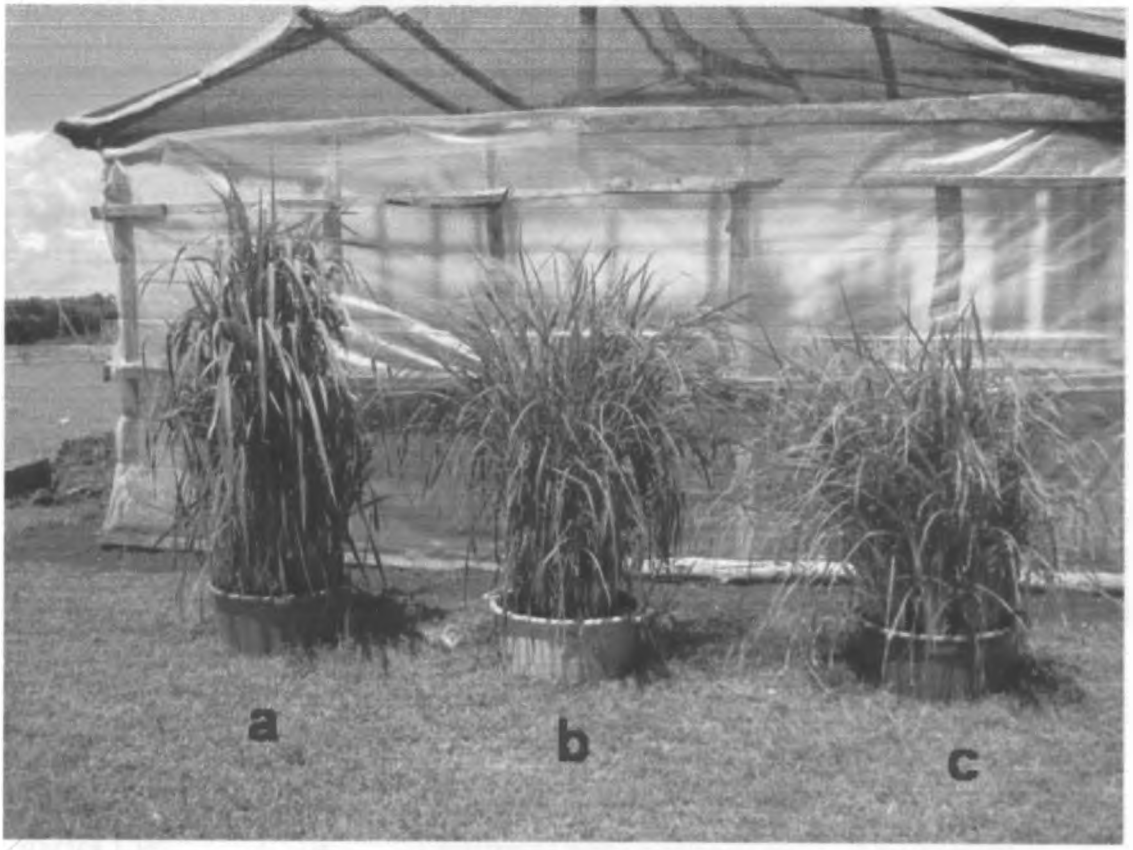
Appendix 6: ANOVA table on panicle length of *O. sativa, O. longistaminata* and the F_1 hybrids

(I) sppname	(J) sppname	Mean Difference (I-J)		Sig.(a)	95% Confidence Interval for Difference(a)
			Upper Bound		Lower Bound
F_1 Hybrids	*O. sativa*	7.31	29.04	0.00	6.40
	O. longistaminata	3.10	24.19	0.00	2.19
O. sativa	F_1 Hybrids	-7.31	28.40	0.00	-8.22
	O. longistaminata	-4.21	0.46	0.00	-5.12
O. longistaminata	F_1 Hybrids	-3.10	0.46	0.00	-4.01
	O. sativa	4.21	0.46	0.00	3.30

Appendix 7: ANOVA table on awn length of *O. sativa, O. longistaminata* and the F_1 hybrids

(I) Plant types	(J) Plant types	Mean Difference (I-J)	Std. Error	Sig.(a)	95% Confidence Interval for Difference(a)	
					Upper Bound	Lower Bound
F_1 Hybrids	*O. longistaminata*	-5.289(*)	0.079	0.000	-5.444	-5.133
	O. sativa	0.032	0.079	0.688	-0.124	0.187
O. longistaminata	F_1 Hybrids	5.289(*)	0.079	0.000	5.133	5.444
	O. sativa	5.320(*)	0.079	0.000	5.165	5.476
O. sativa	F_1 Hybrids	-0.032	0.079	0.688	-0.187	0.124
	O. longistaminata	-5.320(*)	0.079	0.000	-5.476	-5.165

Appendix 8: The three mature plant types; **a)** *O. longistaminata* **b)** F_1 hybrids and **c)** *O. sativa.*



Appendix 9: Unscreened primer pairs

Primer ID	Chromosome location	Allele number	Forward(5'-3')	Reverse(5'-3')
RM14	1	3	CCGAGGAGAGGAGTTCGAC	GTGCCAATTTCCTCGAAAAA
RM220	1	9	GGAAGGTAACTGTTTCCAAC	GAAATGCTTCCCACATGTCT
RM24	1	6	GAAGTGTGATCACTGTAACC	TACAGTGGACGGCGAAGTCG
RM84	1	4	TAAGGGTCCATCCACAAGATG	TTGCAAATGCAGCTAGAGTAC
RM263	2	6	CCCAGGCTAGCTCATGAACC	GCTACGTTTGAGCTACCACG
RM218	3	10	TGGTCAAACCAAGGTCCTTC	GACATACATTCTACCCCCGG
RM241	4	10	GAGCCAAATAAGATCGCTGA	TGCAAGCAGCAGATTTAGTG
RM280	4	4	ACACGATCCACTTTGCGC	TGTGTCTTGAGCAGCCAGG
RM249	5	9	GGCGTAAAGGTTTTGCATGT	ATGATGCCATGAAGGTCAGC
RM289	5	4	TTCCATGGCACACAAGCC	CTGTGCACGAACTTCCAAAG
RM253	6	7	TCCTTCAAGAGTGCAAAACC	GCATTGTCATGTCGAAGCC
RM276	6	6	CTCAACGTTGACACCTCGTG	TCCTCCATCGAGCAGTATCA
RM11	7	7	TCTCCTCTTCCCCCGATC	ATAGCGGGCGAGGCTTAG
RM180	7	2	CTACATCGGCTTAGGTGTAGCA ACACG	ACTTGCTCTACTTGTGGTGAG GGACTG
RM234	7	6	ACAGTATCCAAGGCCCTGG	CACGTGAGACAAAGACGGAG
RM44	8	9	ACGGGCAATCCGAACAACC	TCGGGAAAACCTACCCTACC
RM219	9	7	CGTCGGATGATGTAAAGCCT	CATATCGGCATTCGCCTG
RM258	10	5	TGCTGTATGTAGCTCGCACC	TGGCCTTTAAAGCTGTCGC
RM167	11	4	GATCCAGCGTGAGGAACACGT	AGTCCGACCACAAGGTGCGTT GTC
RM21	11	5	CAGATTGGAGATGAAGTCCTCC	CCAGCAAGCATGTCAATGTA
RM21	11	10	ACAGTATTCCGTAGGCACGG	GCTCCATGAGGGTGGTAGAG
RM235	12	10	AGAAGCTAGGGCTAACGAAC	TCACCTGGTCAGCCTCTTTC