

Distribution of wild rice species and hybridization between cultivated rice and *Oryza longistaminata* in Tanzania

By

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**Declaration**

I declare that this thesis is a record of original work and contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, this thesis contain no material previously published or written by another person except where due reference is made in the text.

Signature of Student: .....

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## **Preface**

This research was performed over 10 months as part of a Master of Plant Biotechnology in accordance with the requirements of the program; the research is presented in the form of a manuscript for submission to a peer-reviewed scientific journal. I have chosen to follow the format of Theoretical and Applied Genetics Journal. My co-authors for the manuscript are Dr Christopher Preston, Dr Juma Kayeke Mohamed and Dr Jenna Malone. Contributions of authors: RK conceived and designed experiments and performed all estimation of hybridization frequencies, prepared tables and figures and wrote large parts of the manuscript. CP supervised my research, read and participated in editing the manuscript. JK reviewed experimental designs used for the screen house experiments, and supervised research activities in Tanzania. JM provided advice on simple sequence repeats (SSR) analysis and interpreted SSR results. All authors read and corrected the complete manuscript. The word count for the manuscript (excluding references and supplementary material) is 5555. Appendix 1 of this thesis contains morphological characteristics of the putative F1 hybrid, cultivated rice and *O. longistaminata* plants recorded from the screen house. Appendix 2 contains number of surveyed rice fields, rice fields infested with wild rice species and frequency of wild rice species in a region. I have followed these instructions. Font: Times New Roman 12. Line numbering: not present for which is similar from journal's instructions in order to satisfy the thesis guidelines for the Master of Plant Biotechnology program.

## **Manuscript**

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## **Abstract**

**Key message:** Three wild rice species were identified in Tanzania, and hybridization between cultivated rice and *O. longistaminata* confirmed. Hybridization frequencies were 2.2% from cultivated rice-*O. longistaminata* and 0.1% from *O. longistaminata*-cultivated rice.

## **Abstract**

Wild rice species of genus *Oryza* are distributed across Asia, Central and South America, Australia and Africa. Wild rice species, such as *O. longistaminata*, with an AA genome can hybridize with cultivated rice under field conditions and produce F<sub>1</sub> hybrids. Such hybridization may cause ecological and evolutionary consequences. An understanding of the distribution of wild relatives of rice and hybridization between cultivated rice and its wild relatives is an important aspect for biosafety in assessing risks caused by gene escape from crop to wild relatives. Despite the presence of five wild rice species in Tanzania, little is known about the distribution of wild rice species that are weeds of rice, or hybridization between cultivated rice and *O. longistaminata* under field conditions. The aim of this study was to generate baseline data on the distribution of wild rice species and hybridization between cultivated rice and *O. longistaminata* under field conditions. A field survey was conducted in 28 rice fields from seven regions and hybridization was determined by morphological and molecular analysis. *Oryza punctata*, *Oryza barthii* and *O. longistaminata* were common wild rice species that are weeds of cultivated rice and hybridization between cultivated rice and *O. longistaminata* were detected under field conditions. Frequencies of hybridization were estimated as 2.2% for crop-to-wild and 0.1% for wild-to-crop. Wild rice species found growing proximity to cultivated rice and hybridization occurs in Tanzania under field conditions with a noticeable frequency from crop-to-wild and wild-to-crop.

**Keywords:** Wild rice species, cultivated rice, biosafety, *O. longistaminata*, hybridization, Tanzania

## **Introduction**

Wild rice species of the genus *Oryza* occur worldwide in rice fields and natural habitats in both tropical and temperate regions (Lu and Snow 2005). Wild rice species can hybridize with genetically compatible cultivated rice and produce F1 hybrids under field conditions. Such hybridization has raised biosafety concerns worldwide. Among the most serious biosafety concerns are environmental consequences caused by transgene escape from genetically modified rice to their compatible wild relatives through gene flow (Conner et al. 2003; Ellstrand et al. 1999; Song et al. 2005). Genetically modified (GM) rice that contains novel traits: resistance to herbicides, greater tolerance of biotic and abiotic stresses, insect and disease resistance and higher yield, high protein content and high beta-carotene (Vitamin A) are under evaluation in confined field trials in Asia, United States of America and Australia (Chen et al. 2011; Xia et al. 2012). In the future, the transgenic rice varieties carrying such traits could be released and commercialized in African countries including Tanzania and used by the rice farmers.

Previous studies have reported occurrence of five wild rice species in Tanzania (Katayama et al. 1987a; Katayama et al. 1987b; Kiambi et al. 2005; Vaughan 1994; Wambugu et al. 2013). These studies concentrated on the *Oryza barthii* conservation of germplasm and analysis of genetic diversity of wild rice species. Of the five wild rice species present in Tanzania, *O. barthii* and *O. longistaminata* share the same AA genome as cultivated rice, however *O. barthii* is a self-pollinating species while *O. longistaminata* is allogamous with a self-incompatibility system, large stigma, long anthers, vigorous creeping and strong underground rhizomes (Ghesquiere 1986; He et al. 2014; Shim 2012). Rhizomes are underground stems that act as means of propagation and dispersal. Furthermore, rhizomes contribute to persistence, competitiveness and weediness in rice fields and invasiveness in natural habitats (Andersson and de Vicente 2010a).

Several studies have identified that *O. longistaminata* can hybridize naturally with cultivated rice when growing sympatrically in the rice field (Andersson and de Vicente 2010a; Kiambi et al. 2005). Cross-pollination and fertilization (hybridization) between these species can produce F<sub>1</sub> hybrids. Further backcross between F<sub>1</sub> hybrids to one of the parents (*O. longistaminata*) could stabilize the hybrids (Vaughan and Morishima 2003). This can facilitate the spread of cultivated rice genes and transgenes in a wild rice population through introgression. If transgenes introduced into a population of wild rice species can enhance fitness they may increase the ability of the transgenic rice hybrids to compete with the wild population in the field under natural conditions (Lu and Snow 2005). Increased fitness of F<sub>1</sub> hybrids may also create new weeds or increased weediness, and cause loss of genetic diversity of wild populations and extinction of existing wild relatives (Conner et al. 2003; Ellstrand et al. 1999; Song et al. 2005).

A number of studies demonstrated that artificial hybridization between cultivated rice and *O. Longistaminata* produced F<sub>1</sub> hybrids. The hybrid plants were identified using morphological characters (Kanya et al. 2012; Tao et al. 2001). However, using morphological characters alone for identification of hybrids is difficult, and it is not always possible to detect and confirm hybrids accurately. Thus for accurate identification of hybrids between crop and wild relatives a more reliable method such as the use of molecular markers is needed. There are different types of molecular markers such as Random Amplified Polymorphic DNAs (RAPDs), Simple Sequence Repeats (SSRs) or microsatellites, Amplified Fragment Length Polymorphisms (AFLPs), Single Nucleotide Polymorphisms (SNPs) and Inter-simple sequence repeat (ISSR) that can be used to identify and confirm hybrids between cultivated rice and wild rice species. Compared with other marker types, SSR markers are very polymorphic, scattered throughout genomes, co-dominant and PCR based and highly amenable to automation and reproducibility (Farooq and Azam 2002; Li et al. 2011). A number of studies have demonstrated that SSR molecular markers are a powerful tool for the identification and confirmation of hybrids



between rice and their respective parents (Shivrain et al. 2007; Song et al. 2004; Sundaram et al. 2008). Thus, this study used SSR molecular marker for identification and confirmation of F<sub>1</sub> hybrids between cultivated rice and *O. longistaminata*.

Knowledge about the distribution of wild rice species in rice growing regions, and hybridization between cultivated rice and wild rice species, is important before introduction and release of GM rice into the environment. It is also an important aspect of biosafety in assessing risks associated with the escape of transgenes from genetically modified rice to wild rice species. Thus, baseline data on the distribution of wild rice species and hybridization between cultivated rice and wild rice species are crucial for regulatory authorities of Tanzania for making plans that can be used to minimize ecological consequences caused by transgene escape from GM rice into wild rice in the future when transgenic rice introduced in Tanzania. Despite the presence of wild rice species distributed in different habitats in Tanzania, little is known about distribution of wild rice species that are weeds of cultivated rice in Tanzania or hybridization between cultivated rice and *O. longistaminata* under field conditions. The objectives of this study were: (1) to identify wild rice species that are weeds of cultivated rice in Tanzania, (2) to determine if natural hybridization occurs between cultivated rice and *O. longistaminata* and, if so, the incidence of hybridization between these species using morphological characters and (3) to confirm F<sub>1</sub> hybrids detected by morphological characters using SSR molecular markers.

## **Materials and Methods**

### **Geographical distribution of wild rice species in rice growing areas**

#### **Study sites and sampling description**

A field survey was conducted to identify wild rice species and their distribution in rice growing regions of Tanzania, focusing on the identification of wild rice types that are weeds of cultivated rice. In April to May 2014 the field survey was carried out in seven rice growing

regions representing major rice growing zones in Tanzania (Table. 1). In all regions, rice production is largely carried out by small-scale farmers. One region was selected from each zone for field survey, except coast zone where two regions were surveyed for the purpose of getting representative samples. In each region, four rice fields infested with wild rice species were surveyed. A total of 28 rice fields were surveyed from seven regions, and wild rice species were identified visually by examining the key characters following a wild rice resource handbook (Vaughan 1994). In each region, records were taken regarding types of wild rice species locality, latitude and longitude. The latitude and longitude of the regions and sites for seed collection were estimated on a map.

**Table 1** Geographical location of surveyed rice growing regions of Tanzania.

Rice growing zone	Region	Latitude	Longitude
Lake Victoria	Mwanza	02°30'S	32°58'E
Northern	Manyara	4° 12' S,	35° 45' E
Southern highlands	Mbeya	08°54'S	33°29'E
Central	Tabora	5°2'S	32°50'E
Eastern	Morogoro	08°0'S	37°0'E
Coast	Zanzibar Urban/West	06°09'50"S	39°11'52"E
Coast	Zanzibar Central/South	-6° 15' 0"S	39°28' 00" E

### Collection of seed samples

Seed samples were collected from two regions where wild rice and cultivated rice were mature. Within each region, four representative rice fields were selected based on the rice field infested with wild rice species. A total of 8000 seeds of each of *O. longistaminata* and cultivated rice growing sympatrically were randomly collected from each region. *O. longistaminata* seeds collected from the edge of ponds and cultivated rice collected from the rice fields free from *O. longistaminata* were used for comparison in subsequent experiments (Table 2).

**Table 2** Seed samples of four species used in the present study and collection sites.

Seed sample	No. of seeds	Region	Locality	Latitude	Longitude
Cultivated rice	2000	Mwanza	Nyakahako	2° 27' 36"	32° 40' 12"
	2000	Mwanza	Nganza	2° 36' 00"	32° 53' 00"
	4000	Manyara	Kisangaji	3° 55' 02"	35° 43' 56"
<i>O. longistaminata</i> from rice fields	2000	Mwanza	Nyakahako	2° 27' 36"	32° 40' 12"
	2000	Mwanza	Nganza	2° 36' 00"	32° 53' 00"
	4000	Manyara	Kisangaji	3° 55' 02"	35° 43' 56"
Cultivated rice from rice field infested with <i>O. longistaminata</i>	2000	Mwanza	Nyakahako	2° 27' 36"	32° 40' 12"
	2000	Mwanza	Nganza	2° 36' 00"	32° 53' 00"
	4000	Manyara	Kisangaji	3° 55' 02"	35° 43' 56"
<i>O. longistaminata</i>	8000	Mwanza	Nganza	2° 36' 00"	32° 53' 00"

### Determination of hybridization between cultivated rice and *O. longistaminata*

For the determination of hybridization between cultivated rice and *O. longistaminata*, two experiments were conducted under screen house conditions at Mikocheni Agricultural Research Institute in Dar es Salam, Tanzania in 2014. The first experiment was carried out to detect F<sub>1</sub> hybrids from the crop-to-wild rice hybridization. Five thousand seeds of *O. longistaminata* that was growing sympatric with cultivated rice in the rice fields were selected randomly from the 8,000 seeds collected in the rice fields. *Oryza longistaminata* seeds collected from the edge of ponds and cultivated rice collected from the rice fields free from *O. longistaminata* infestation were planted for comparative controls. Seeds were germinated in separate small plastic basins filled with soil. After three weeks, 600 seedlings of each of the two plant species were planted in each plot (Fig. S1). The plots were filled with sandy clay soil collected from rice fields free from the rice crop. The transplanted plants in each plot were flooded with water to a depth of 12cm and maintained until the plants reached maturity. Plants were kept weed-free by hand-weeding. The experiment was laid out in a randomized completely block design with four replicates. At maturity, a total of 600 of each plant species were selected randomly and tagged. Plant height, flag leaf length, flag leaf width and number of tillers per plant were measured from the selected plants. The rice descriptors published by International Plant Genetic Resources Institute (2007) were used for characterization of plant

species (Table 3). The mean values of each trait for each plant species were calculated and compared.

**Table 3** Descriptors used to characterize cultivated rice, putative F<sub>1</sub> hybrid and *O. longistaminata* plants (Institute 2007)

Descriptor	Description
Plant height	Measured from soil surface to the tip of tallest panicle (cm)
Flag leaf length	Measured from the ligule to the tip of the blade (cm)
Flag leaf width	Measured at the widest portion of the leaf (cm)
Tillers per plant	Number of tillers counted per each plant
Maturity	Date on which 80% of the grains on panicles are fully ripened

A second experiment was carried out to detect putative F<sub>1</sub> hybrids from wild-to-crop hybridization. Five thousand seeds of cultivated rice were randomly selected from the 8,000 collected from the rice fields infested with *O. longistaminata*. A similar number of *O. longistaminata* seeds collected from the edge of ponds and cultivated rice grown in rice fields free from *O. longistaminata* were used as controls. The seeds were sown into separate plastic pots. After three weeks, 3600 seedlings were transplanted in four plots at a density of 600 plants/plot. The experiment was laid out in complete randomized design with four replications (Fig. S2). Plants were flooded with water to a depth of 12 cm and maintained until maturity. At maturity, all 3600 plants species were uprooted from the soil; their roots were washed with clean water and examined for the presence of rhizomes. Putative F<sub>1</sub> hybrids were detected on the basis of the presence of the rhizomes.

#### **Assessment of hybridization frequency by morphological characteristics**

Hybridization frequencies from crop-to-wild and wild-to-crop hybridization were estimated using formula described by Loureiro et al (2006) and Bae et al (2013) with few modifications.

$$\text{Hybridization frequency (\%)} = \frac{\text{Number of putative F}_1 \text{ hybrids}}{\text{Total number of plants}} \times 100$$

### **Confirmation of hybridization using SSR markers**

#### **Extraction of DNA**

Total genomic DNA was isolated from 50 fresh leaf samples of detected putative F<sub>1</sub> hybrids, 4 samples of cultivated rice and 4 samples from *O. longistaminata* using the Isolate Plant DNA Mini Kit (BIOLINE, London, UK).

#### **SSR PCR amplification**

The extracted DNA was amplified in polymerase chain reactions using SSR primer pair RM515 (forward: TAGGACGACCAAAGGGTGAG/reverse: TGGCCTGCTCTCTCTCTC) as reported by Ren et al (2003). PCR was performed in a total volume of 25 μl reaction mixture containing 100 ng of template DNA, 5 μl MyFi reaction buffer, 20 μM (1 μl) of SSR primer pairs and 1 μl of MyFi DNA polymerase. DNA amplification was performed in a Gene Touch Thermal Cycler, programmed for an initial 1 minute at 95°C, followed by 35 cycles of 15 seconds at 95°C, 15 seconds at 55°C and 15 seconds at 72°C for the final extension. The amplified products were separated by electrophoresis on 4 % agarose gel. The gel was stained with SYBR<sup>®</sup> Safe DNA gel stain (Invitrogen), and photographed under ultraviolet light using a gel documentation system. Since SSR markers are codominant, both alleles (one from cultivated rice and one from *O. longistaminata*) will be present in any F<sub>1</sub> hybrids.

#### **Assessment of hybridization frequency by molecular analysis**

Hybridization frequencies confirmed via SSR marker analysis were determined as before.

The final hybridization frequency was estimated using the formula described Chen et al (2004).

$$\text{Hybridization frequency} = \frac{\text{Hybridization by morphological characterization} \times \text{Hybridization frequency by SSR analysis}}{100}$$

### Data analysis

Data were analysed using Genstat software (Mead et al. 2002; Payne 2009). Data on the morphological quantitative traits and number of detected putative F<sub>1</sub> hybrids were subjected to one-way analysis of variance for unbalanced data. Means of each morphological character of the putative F<sub>1</sub> hybrids and parents were separated by Fishers protected tests at P < 0.05.

### Results

#### Geographical distribution of wild rice species in rice growing areas in Tanzania

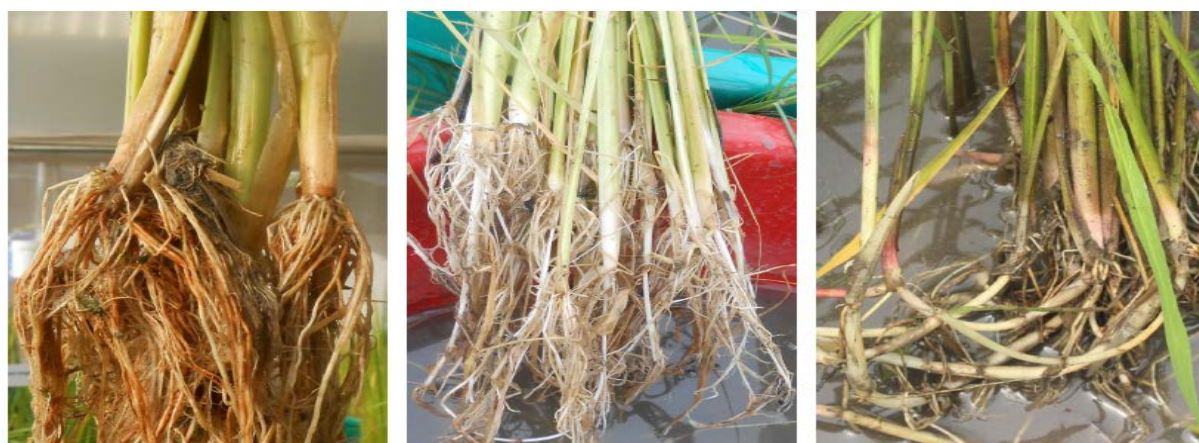
Results of the field survey showed that 22 of 28 (78.57%) surveyed rice fields were infested with wild rice species while 6 rice fields were free from wild rice species. Three types of wild rice species: *O. punctata*, *O. barthii* and *O. longistaminata* (Fig.S3 (a-c)) were found growing in and at the edge of rice fields as weeds of cultivated rice. Of the three types of wild rice species, *O. longistaminata* was widely distributed in six surveyed rice growing regions (Mwanza, Tabora, Manyara, Zanzibar Urban/West, Mbeya and Morogoro). *Oryza punctata* was identified in Zanzibar Central/South, Manyara and Morogoro while *O. barthii* was only found in Tabora region. Thus, wild rice species co-exist with cultivated rice and are found growing in proximity (Fig.S4 (a-c)).

## Determination of hybridization between cultivated rice and *O. longistaminata* by morphological characteristics

In the cultivated rice-to-wild rice hybridization experiment, 44 of 600 plants characterized using morphological characters were determined to be putative F<sub>1</sub> hybrids of cultivated rice and *O. longistaminata* (Table 4). Putative F<sub>1</sub> hybrid plants detected from crop-to-wild rice hybridization were significantly different from both cultivated rice and *O. longistaminata* as indicated by the one-way analysis of variance (Table 5). For hybridization from wild rice to cultivated rice, 93 of 3600 grown plants examined for the presence of the rhizome trait were determined as putative F<sub>1</sub> hybrids. Putative F<sub>1</sub> hybrid plants had intermediate and weak rhizomes compared to *O. longistaminata*. The roots of putative F<sub>1</sub> hybrid plants contained multiple nodes below the node of the seed (Figure 1). A higher incidence of hybridization was found 7.33% in cultivated rice-to-wild rice hybridization than in wild rice-to-cultivated rice was 2.58% (Table 4).

**Table 4** Detected putative F<sub>1</sub> hybrids and incidence of hybridization between cultivated rice and *O. longistaminata*

Hybridization	Plants examined	Detected F <sub>1</sub> hybrids	% of F <sub>1</sub> hybrids
Cultivated rice-to- <i>O. longistaminata</i>	600	44	7.33
<i>O. longistaminata</i> -to-cultivated rice	3600	93	2.58



**Fig. 1** Cultivated rice (left), putative F<sub>1</sub> hybrids with rhizomes containing multiple nodes below the node of the seed (middle) and *O. longistaminata* (right).

**Table 5** Statistical comparison of the morphological traits between putative F<sub>1</sub> hybrids, cultivated rice and *O. longistaminata* and. (N = 600 individuals from 4 replicates). Statistical analysis was based on one-way analysis of variance for unbalanced data because sample sizes for treatment combinations used in the experiment were not equal.

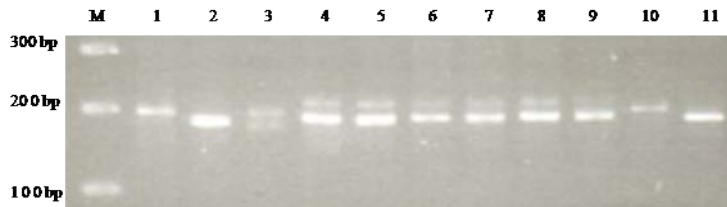
Plant species	Morphological traits			
	Plant height (cm)	Flag leaf length (cm)	Flag leaf width (cm)	Tillers per plant (No)
Cultivated rice	86.6 <sup>a</sup>	34.3 <sup>a</sup>	1.1 <sup>a</sup>	2.0 <sup>a</sup>
<i>O. longistaminata</i>	95.4 <sup>a</sup>	35.4 <sup>a</sup>	1.2 <sup>a</sup>	6.0 <sup>b</sup>
Putative F <sub>1</sub> hybrids	113.8 <sup>b</sup>	53.1 <sup>b</sup>	1.5 <sup>b</sup>	7.0 <sup>c</sup>

Means followed by the same letters in columns are not significantly different at 0.05 level of significance.

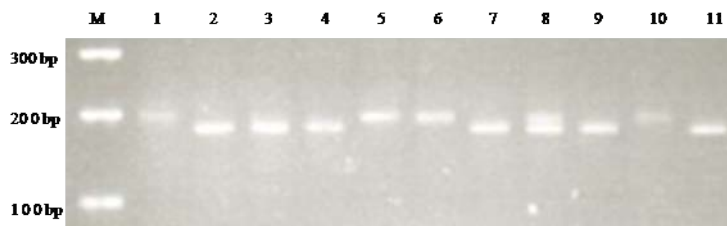
### Confirmation of hybridization using SSR markers

Results of SSR analysis show that 7 of 23 plants examined by the SSR marker RM515 were confirmed to be F<sub>1</sub> hybrids from cultivated rice to *O. longistaminata* hybridization. The PCR amplification with SSR primer pair RM515 pair produced two amplicons of DNA, one corresponding to each parent (Lanes 3-9 Fig. 2). The cultivated rice and *O. longistaminata* had one band of DNA with clear variability in size (Lanes 1, 10 and 2, 11 Fig. 2). For *O. longistaminata*-to-cultivated rice hybridization, 1 plant was found to be F<sub>1</sub> hybrid (Lane 8 in Fig. 3). Two plants (Lanes 5-6 Fig. 3) had one amplicon of DNA corresponding to cultivated rice (parent). Four plants (Lanes 3, 4, 7, 9 Fig. 3) had one amplicon of DNA corresponding to *O. longistaminata*. Proportions of hybrids among the plants analyzed by SSR marker RM 515 pair were 30.43% and 4.35% from cultivated rice-to-*O. longistaminata* and from *O. longistaminata*-to-cultivated rice hybridization, respectively.





**Fig. 2** SSR amplification products generated by the primer RM 515. Lanes 1 and 10, cultivated rice (male); Lanes 2 and 11, *O. longistaminata* (female); and Lanes 3-9 F<sub>1</sub> hybrids between cultivated rice and *O. longistaminata*. M = DNA marker.



**Fig.3** SSR amplification products generated by the primer RM515. Lanes 1, 5, 6 and 10, cultivated rice; Lanes 2 3, 4, 7, 9 and 11, *O. longistaminata*; and Lane 8, F<sub>1</sub> hybrids between cultivated rice and *O. longistaminata*. M = DNA marker.

## Discussion

### Geographical distribution of wild rice species in rice growing areas in Tanzania

Wild rice species that are weeds of cultivated rice were found in and around rice fields in all seven surveyed regions of Tanzania. The present study found wild rice species in localities where they have not been reported in the previous literature. The spread of wild rice species in rice growing regions might be attributed to the use of cultivated rice seeds contaminated with the seeds of wild rice species (Goulart et al. 2014). In addition, other factors such as flooding, machinery, irrigation and bird dispersion might contribute to the wild rice seed movement between rice fields (Goulart et al. 2014). The findings of this study concur with the findings of the previous studies (Agnoun et al. 2012; Katayama et al. 1987b; Kiambi et al. 2005; Vaughan 1994), which demonstrated that wild rice species were widely distributed in lakes, ponds, along rivers, in natural vegetation, and at the edge of and/or within farmer's rice fields.

These wild rice species include *O. punctata*, *O. barthii* and *O. longistaminata*. However, findings of the present study differ from previous studies in terms of the focus of collection and habitats of the wild rice species. The current study focused on the assessment of wild rice species in rice field habitats only and clearly showed the distribution of wild rice species that are weeds of cultivated rice in rice growing areas of Tanzania.

### **Determination of hybridization between cultivated rice and *O. longistaminata* using morphological characters**

In addition to the assessment of wild rice species distribution, determination of hybridization between wild rice and cultivated rice is another important factor in the assessment of gene flow considerations. In the crop-to-wild rice hybridization study, high hybridization frequency was detected by morphological characters (height and flag leaf length). Identified putative F<sub>1</sub>s were taller, had wider flag leaf length, flag leaf width and more tillers than cultivated rice and *O. longistaminata*. Formation of putative F<sub>1</sub> hybrids with quantitative traits significantly exceed those comparative controls (cultivated rice and *O. longistaminata*) which might be attributed to overdominance or heterosis due to multiple additive genes which occurred under field conditions (Hua, Xing et al. 2003). In *O. longistaminata* to cultivated rice, putative F<sub>1</sub> hybrid plants had intermediate and weak rhizomes compared to *O. longistaminata*. The formation of putative F<sub>1</sub> hybrids with an intermediate rhizome trait shows that the putative F<sub>1</sub> hybrids inherited this trait from *O. longistaminata*. Results of this study correspond to the findings reported by Tao et al (2001) who found that F<sub>1</sub> hybrids had intermediate rhizomes trait inherited from *O. longistaminata*.

### **Confirmation of hybridization using SSR markers**

The SSR amplification products generated by the primer RM515 showed that F<sub>1</sub> hybrids were confirmed from both crop-to-wild and wild-to-crop hybridization. Confirmation of F<sub>1</sub> hybrids clearly indicates the occurrence of hybridization under field conditions. Results of the present

study demonstrate that a higher frequency of hybridization was obtained from cultivated rice to *O. longistaminata* than from *O. longistaminata* to cultivated rice. This might be attributed to the same heading dates and synchronization of flowering time of the cultivated rice and *O. longistaminata*, proximity of the two species and frequencies of the pollen flow (Song, Lu et al. 2003, Chen, Lee et al. 2004). Other factors could be a high proportion of exerted stigma of *O. longistaminata* than cultivated rice (He et al. 2014; Shim 2012). Results of the present study are in agreement with the reports of the previous studies (Andersson and de Vicente 2010; Kiambi et al. 2005). These studies reported that the cultivated rice and *O. longistaminata* can hybridize under natural conditions. Also, the findings of this study on the frequency of hybridization from crop-wild hybridization are supported by the reports of the previous studies (Ellstrand et al. 1999; Klinger and Ellstrand 1994). These studies show that rates of hybridization from crop-to-wild hybridization exceeds 1% when the crop and wild relatives occur in close proximity under field conditions.

Low hybridization frequency from the *O. longistaminata* to cultivated rice could be due to different of heading dates, unsynchronized flowering time and different in height between cultivated rice to *O. longistaminata* (Chen et al. 2003). Results of the present study are in agreement with the reports of the previous studies (Andersson and de Vicente 2010b; Kiambi et al. 2005). These studies reported that the cultivated rice and *O. longistaminata* can hybridize under natural conditions. Also, the findings of this study concur with the previous report of Lu (2008) who found that hybridization through pollen-mediated gene flow is two-directional event under natural conditions. Confirmation of F<sub>1</sub> hybrids clearly indicates that hybridization between cultivated rice and *O. longistaminata* occurs in Tanzania under field conditions. However, results of this study were derived from two experiments carried out in screen house conditions with a limited number of plants in small-scale plots, and thus might not give correct reflection of the hybridization rate in general. The study by Chen et al (2004) obtained hybridization frequency ranged from 1.21 -1.19% under field conditions when

sample of 2246 seedlings were analysed by SSR markers. Further experiments are required to study hybridization rates between cultivated rice and *O. longistaminata*, which may include larger representation of plants stands within the population under cultivation conditions.

### **Implications**

Results of this study provide biosafety regulatory authorities of Tanzania with information about distribution of wild rice species that are weeds of cultivated rice, and hybridization frequencies between cultivated rice and *O. longistaminata*. The information will be useful for evaluating the ecological effects of new transgenic varieties containing herbicide resistance trait that might be introduced in Tanzania and used by farmers. In addition, data from this study can be used by the regulatory authorities in making decisions about field-testing, commercialization, and possible monitoring of transgenic rice when introduced in Tanzania in the future. Furthermore, results of the present study are the stepping stone which can help biosafety regulators and risk assessors to plan for a tired process of assessment for environmental risks caused by transgene escape from GM rice to its wild relatives. Moreover, findings of this study can assist weed scientists to develop effective strategies for control of *O. longistaminata*.

The findings of this study demonstrate that three wild rice species co-exist in the rice fields as weeds of cultivated rice in seven rice growing regions of Tanzania; and hybridization between cultivated rice and *O. longistaminata* produced F<sub>1</sub> hybrids under field conditions. The F<sub>1</sub> hybrids of cultivated rice and *O. longistaminata* can serve as a bridge for gene flow to other related wild rice species if they co-exist in the same rice fields. Since F<sub>1</sub> hybrids were produced, fitness estimation through performance comparison of F<sub>1</sub> hybrids with their parental species *O. longistaminata* and cultivated rice is focus of future research. The present study identified wild rice species that are weeds of cultivated rice in Tanzania, and natural hybridization between cultivated rice and perennial wild rice species, *O. longistaminata* occurred in rice growing regions.

Therefore, management plan should be considered when introducing genetically modified rice in rice growing regions where wild rice species, *O. longistaminata* grow sympatrically with cultivated rice.

**Author Contributions** RK conceived and designed experiments and performed all statistical analyses, prepared tables and figures and wrote large parts of the manuscript. CP supervised the research, read and participated in editing the manuscript. JK reviewed experimental designs used for the screen house experiments, and supervised research activities in Tanzania. JM provided advice on SSR analysis and interpreted SSR results.

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**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical standards** The experiments performed in this study comply with the current laws of the United Republic of Tanzania where they were performed.

## **Supplemental materials**

### **Theoretical and Applied Genetics**

Distribution of wild rice species and hybridization between cultivated rice and *Oryza longistaminata* in Tanzania

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Block 1	Block 2	Block 3	Block 4
1 Cultivated rice	5 Rice crop-to-wild rice F1	9 <i>O. longistaminata</i>	13 Rice crop-to-wild rice F1
2 Wild rice-to-rice crop F1	6 Cultivated rice	10 Rice crop-to-wild rice F1	14 Cultivated rice
3 Rice crop-to-wild rice F1	7 <i>O. longistaminata</i>	11 Wild rice-to-rice crop F1	15 <i>O. longistaminata</i>
4 <i>O. longistaminata</i>	8 Wild rice-to-rice crop F1	12 Cultivated rice	16 Wild rice-to-rice crop F1

**Fig. S1** Randomized complete block design (RCBD) with four replications used for cultivated rice to *O. longistaminata* (crop-to-wild rice) hybridization study under screen house conditions. Numbers in upper left hand corner are plot numbers. One block = one replicate.

Rep1	1 Cultivated rice	2 Wild rice-to-rice crop F1	3 <i>O. longistaminata</i>
Rep2	4 <i>O. longistaminata</i>	5 Cultivated rice	6 Wild rice-to-rice crop F1
Rep3	7 Cultivated rice	8 <i>O. longistaminata</i>	9 Wild rice-to-rice crop F1
Rep4	10 Wild rice-to-rice crop F1	11 Cultivated rice	12 <i>O. longistaminata</i>

**Fig. S2** Complete randomized design (CRD) with three treatments replicated four times was used for *O. longistaminata* to cultivated rice (wild rice- to-crop) hybridization study under screen house conditions. Numbers in upper left hand corner are plot numbers.



**Fig. S3** (a-c) Rice fields infested with wild rice species in Tanzania. (a) *Oryza barthii*





(b) *Oryza longistaminata*



(c) *Oryza punctata*



**Fig. S4** (a-c) Rice fields infested with wild rice species in Tanzania. (a) Rice field infested with *O. barthii* in Nzega district in Tabora region, Tanzania



(b) Rice field infested with *O. longistaminata* in Babati district in Manyara region, Tanzania



(c) Rice field infested with *O. punctata* at Uzini village, Zanzibar Island, Tanzania.

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## Appendices

### Appendix 1: Morphological traits measured from three plant species.

Data on the morphological characteristics (plant height, tiller numbers flag leaf length width) of cultivated rice, *O. longistaminata* and Putative F<sub>1</sub> hybrids were recorded at maturity stage under screen house conditions at Mikocheni Agricultural Research Institute in Dar es Salaam, Tanzania in August 2014. Six hundred of each plant species in each block were averaged and presented in Table 6.

**Table 6** Morphological characteristics (plant height, tiller numbers flag leaf length width) recorded from cultivated rice, putative F<sub>1</sub> hybrids and *O. longistaminata* plants.

Plant species	Block	Plant height (cm)	Flag leaf length (cm)	Flag leaf width (cm)	Tillers per plant (No)
Cultivated rice	1	95.3	30.6	1.2	3
Cultivated rice	2	83.4	35.0	1.2	2
Cultivated rice	3	87.3	36.0	1.1	2
Cultivated rice	4	80.4	35.7	1.1	2
Putative F <sub>1</sub> hybrids	1	115.2	54.2	1.5	9
Putative F <sub>1</sub> hybrids	2	111.2	52.6	1.6	8
Putative F <sub>1</sub> hybrids	3	117.1	51.9	1.6	6
Putative F <sub>1</sub> hybrids	4	111.9	53.7	1.5	6
<i>O. longistaminata</i>	1	88.8	35.8	1.4	8
<i>O. longistaminata</i>	2	101.2	35.2	1.2	6
<i>O. longistaminata</i>	3	94.4	33.8	1.2	4
<i>O. longistaminata</i>	4	97.4	36.7	1.2	5

**Appendix 2:** Surveyed rice fields from seven rice growing regions in Tanzania

During field survey in April to May, four rice fields were visited to assess types of wild rice species that are weeds of cultivated rice. A total of 28 rice fields were surveyed. 22 of 28 rice fields were infested with wild rice species. The percentage frequency of wild rice species was computed and presented in Table 7.

**Table 7** Surveyed rice fields, rice fields infested with wild rice species and percentage frequency of wild rice species in a region

Region	No. of fields surveyed	No. of fields with wild rice species	% Frequency of wild rice species
Mwanza	4	4	100
Manyara	4	4	100
Morogoro	4	4	100
Mbeya	4	3	75
Tabora	4	4	100
Zanzibar Central/South	4	1	25
Zanzibar Urban/West	4	2	50
Total	28	22	78.58%