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Gene Flow Between the Wild Rice Species (*Oryza longistaminata*) and Two Varieties of Cultivated Rice (*Oryza sativa*) in Kilombero District, Tanzania

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Abstract

The direction and rate of gene flow between the perennial wild rice species, *O. longistaminata* and cultivated rice, *O. sativa* was investigated using nine Microsatellite (SSR) markers. The study involved characterization of parental lines of two cultivated rice varieties and *O. longistaminata* and their F1 progenies. Presence of crop-specific alleles in the wild species or vice-versa was used as an indicator of occurrence of gene flow between the two species. The study revealed that gene flow between *O. longistaminata* and each of the two varieties of *O. sativa* occurs naturally. The direction of gene flow was mainly from the cultivated to wild species. The rate (frequency) of gene flow was higher from cultivated (*O. sativa*) to wild rice (*O. longistaminata*) than from *O. longistaminata* to *O. sativa* and varied with cultivated rice variety. Higher rate of gene flow from cultivated to wild species could be due to floral structure and out-crossing nature of the wild species (*O. longistaminata*). Gene flow between *O. longistaminata* and *O. sativa* is likely to change genetic integrity of natural populations of the two species in areas where they occur sympatrically. However, isolation by distance can help to control gene flow between these species.

Keywords: Gene flow, Microsatellite markers, *Oryza longistaminata*, Wild rice

Introduction

In many parts of Tanzania the cultivated rice (*Oryza sativa)*, which is an annual plant species grows sympatrically with its wild relatives including *Oryza longistaminata*. The *O. longistaminata*, which is the only perennial wild rice species in Tanzania (Mangosongo et al. 2019), is a source of agronomically useful traits that can be used in the rice improvement programmes (Melaku et al. 2019, Mangosongo et al. 2020). Moreover, *O. longistaminata* is one of the common weeds in the cultivated rice fields (Kiambi et al. 2005, Rodenburg and Johnson 2009, Melaku et al. 2013, Melaku et al. 2019). Although the two species (*O. sativa* and *O. longistaminata*) differ in their breeding systems, they are sexually compatible (Kiambi et al. 2008). The *O. sativa* is a self-pollinated species, whereas *O. longistaminata* is a cross-pollinated species. Sympatric existence (coexistence) of the two species that are sexually compatible increases the chance for occurrence of gene flow between them when they have synchronized flowering periods (Arias and Rieseberg 1994, Oppong et al. 2019). Therefore, coexistence of *O. sativa* and its wild relative, *O. longistaminata* is likely to result into exchange of genetic material (gene flow) between them.

Gene flow is the movement of genetic information between two organisms in which one organism serves as the donor and the other serves as the recipient (Oppong et al. 2019). The incoming genes may then be incorporated in the gene pool of the receiving population through the process known as

introgression (Harrison and Larson 2014, Zhang et al. 2022). Introgression involves incorporation of alleles from two different parental taxa to form a zygote which develops into a new individual called a hybrid, and the process by which a hybrid is formed is termed hybridization (Harrison and Larson 2014, Culicchi 2022). Gene flow between plant species can occur through three different ways or mechanisms, namely pollen-mediated gene flow, seed-mediated gene flow (Oppong et al. 2019, Zhang et al. 2022) and vegetative propagule-mediated gene flow (Oppong et al. 2019). The present study assessed pollen-mediated gene flow, which occurs through outcrossing or movement of pollens. Occurrence of gene flow and hybridization between two species is usually manifested by morphological intermediacy and can be confirmed by molecular markers (Rieseberg and Ellstrand 1993, Albert et al. 1997), which can detect hybrids regardless of their morphological intermediacy to their parents. The process of gene flow through pollen starts with the arrival of pollen to the focal population, thus gene flow through pollen between two taxa can occur only when the distance between them is short to allow the arrival of the pollen grains (Oppong et al. 2019). A study by Kanya et al. (2009) on dispersal distance of rice (*O. Sativa* L.*)* pollen conducted at the Tana River delta in the Coast province of Kenya revealed that the maximum dispersal distance of the rice pollens is about 250 metres. The direction of pollen dispersal is determined by the prevailing wind direction (Kanya et al. (2009). Literature further shows that wind speed has great influence on pollen dispersal (Song et al. (2003).

Gene flow has the potential of increasing the genetic diversity of a population by adding valuable alleles to a population (Ellstrand 2014). The effect that gene flow and introgression can have on populations depends on the degree or rate and direction of gene movement (Ellstrand 2014), hence it is important to understand the rate and direction of gene between the two species. Gene flow from the cultivated to wild species can add new genes into wild species populations, which can then re-assort into novel combinations that may have a substantial impact on the evolution of wild populations (Arriola and Ellstrand 1996, Ellstrand et al. 1999). In addition, aggressive spreading of hybrid swarms with better ecological fitness can lead to local extinction of endangered wild species' populations (Kiang et al. 1979). According to Heredia-Pech et al. (2022) recurrent events of gene flow and introgression between cultivated species and their wild relatives can generate the existence of wild-weedy-crop complexes.

Although several studies have demonstrated that hybridization in the rice crop-weed-wild relative complex takes place (Ellstrand et al. 1999, Kiambi et al. 2008), the direction and actual rate of gene flow varies with the cultivated rice variety involved (Song et al. 2003) as well as with the microclimatic conditions of the area. Knowledge on the direction and rate of gene flow is essential for conservation and proper management or control of gene flow between the species. However, before the present study no comprehensive study had been conducted in the study area to assess the direction and rate of pollen-mediated gene flow between the two cultivated rice varieties and wild rice species, *O. longistaminata*. The present study used microsatellite markers to assess the direction and rate of pollen-mediated gene flow between the wild rice species (*O. longistaminata* and two rice (*O. sativa*) varieties that were cultivated in the study area. The two cultivated rice varieties were *O. sativa* var. kalamata and var. zambia.The microsatellite markers, which are also known as Simple Random Repeats (SSR) markers were preferred in this study due to their co-dominance nature, technical simplicity, relatively low cost, high power of genetic resolution and high polymorphism in plant species (Zhou et al. 2003, Semagn et al. 2006, Melaku et al. 2013, Mangosongo et al. 2020). The markers used in the present study have been widely used in rice population studies (Mangosongo et al. 2020).

Materials and Methods

The study sites

The study on gene flow between wild rice species, *O. longistaminata* and the cultivated rice species, *O. sativa* was carried out in Kilombero district. The study involved assessment of natural gene flow between *O. longistaminata* and two varieties of *O. sativa* that were grown in the study area. These were *O. sativa* var. zambia and var. kalamata. The study consisted of two experiments and was conducted in two different rice fields in which *O. longistaminata* were growing sympatrically with each of the two varieties of *O. sativa* as a weed. The two fields were about five hundred metres apart.

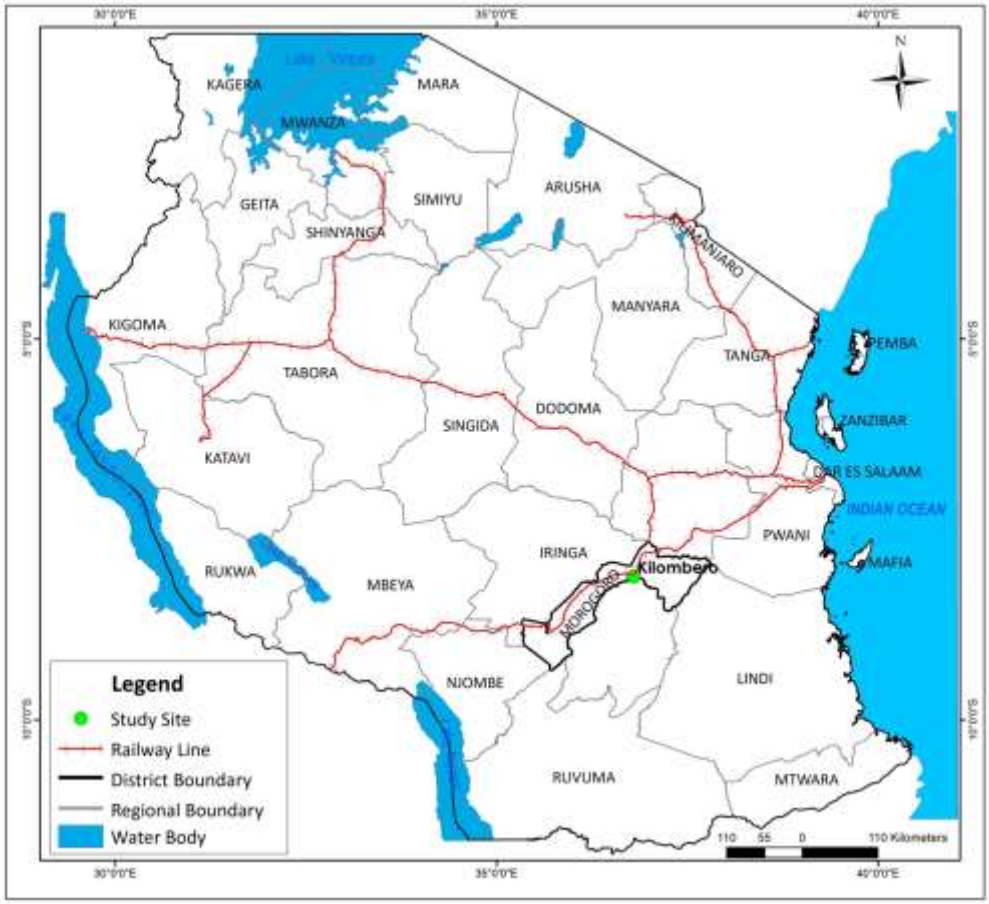

Figure 1: The map of Tanzania showing the location of study area

Collection of leaf samples

In each site, a reconnaissance survey was conducted in the rice fields where each of the two cultivated rice varieties and *O. longistaminata* coexisted (were growing sympatrically) in order to identify and mark individual plants of the two species that overlapped their flowering periods. In each site, only individuals of the two species that were found to have synchronized their flowering periods were monitored and considered for sampling. At each sampling point a leaf sample of *O. longistaminata* individual and two samples from two *O. sativa* individuals were collected from the marked plants. At each point of collection of *O. longistaminata* two *O. sativa* samples from two *O. sativa* individuals located in different directions within 5-metre-distance around the point of collection of *O.*

longistaminata were collected. The collected leaf samples were preserved with silica gel into zip-locked plastic bags. Rice seeds on the individual plants from which leaf samples had been collected were left *in situ* to allow their maturation and ripening.

After ripening, a total of one hundred seeds of *O. longistaminata* and one hundred seeds of the two cultivated rice varieties (*O. sativa* var. kalamata and var. zambia) were collected from each individual plant (a plant from which leaf samples had been collected) in each experimental field plot. The collected seeds from each experiment were dried by exposing them to full sunlight for three days and then stored at room temperature for two months in order to break seed dormancy. After two months, fifty seeds of *O. longistaminata* and fifty seeds of each of the two *O. sativa* varieties were randomly selected for germination onto the plastic basins containing soil. However, *O. longistaminata* seeds showed poor germinability as most of the seeds could not germinate. In the experiment on gene flow between *O. longistaminata* and *O. sativa* var. zambia, only ten seeds of *O. longistaminata* and forty nine seeds of *O. sativa* var. zambia germinated into seedlings, while in the experiment on gene flow between *O. longistaminata* and *O. sativa* var. kalamata only eleven seeds of *O. longistaminata* and forty seven seeds of *O. sativa* var. kalamata germinated into seedlings. In the first experiment, the sample size consisted of one parental *O. longistaminata* and two *O. sativa* var. zambia, the ten seedlings of *O. longistaminata* and nineteen randomly selected seedlings of *O. sativa* var. zambia. In the second experiment, the sample size consisted of the one parental *O. longistaminata* and two *O. sativa* var. kalamata, the eleven seedlings of *O. longistaminata* and eighteen randomly selected seedlings of *O. sativa* var. kalamata. Thus, the sample size in the present study consisted of samples from two parental *O. longistaminata* and four parental *O. sativa* as well as fifty eight samples from the progenies of the two species collected from the seedlings. Two to three young leaf samples were collected from each individual progeny (seedling) of the two species about fifty days after germination. The collected leaf samples were then used for DNA extraction and further genetic analysis using nine SSR markers.

DNA extraction

Genomic DNA was extracted from young leaf tissue using protocol adopted from Dellaporta et al. (1983), with minor modifications. 100 mg of leaf tissue was powdered by grinding in liquid nitrogen and incubated in extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 100 mM NaCl and 1.25% (w/v) SDS) containing 200 µL of 5 M potassium acetate at 65 °C for 30 minutes. The slurry was extracted with 0.8 µL of chloroform-isoamyl alcohol (24:1 v/v), and the emulsion was centrifuged at 5000 rpm at 4 °C for 15 minutes. Extracted DNA was precipitated from supernatant with 950 g/L alcohol and washed with 700 g/L alcohol three times. After drying, DNA was dissolved in Tris-EDTA, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA) containing 100 µL RNAase. DNA samples were then stored at -20 °C until when they were used.

Polymerase Chain Reaction (PCR)

The PCR was carried out in a programmable thermal cycler (Applied Biosystems 9700) using nine SSR primer pairs and Touchdown PCR protocol. The total reaction volume was 10 µl, containing 2.5 µl of genomic DNA and 7.5 µl of premix solution. The premix solution contained 5.82 µl of distilled water, 1.0 µl of 10x PCR buffer, 0.2 µl of dNTP, 0.2 µl of forward primer, 0.2 µl of reverse primer and 0.08 µl of Taq polymerase. Amplification reactions consisted of 35 temperature cycles, which were initiated by 3 minutes of pre-denaturation at 95 °C followed by denaturation at 94 °C for 30 seconds, annealing at 64 °C for 1 minute and extension at 72°C for 30 seconds (in 10 cycles). Then the content was denatured at 94 °C for 30 seconds, annealing at 54 °C for 1 minute, extension at 72 °C for 30 seconds (in 25

cycles). A final extension step at 72 °C for 20 minutes was performed after 35 cycles. Finally, the content was cooled at 10°C indefinitely. Fragment analysis of the PCR amplified products was performed using Gene Mapper ID-X, version 1.1 (Applied Biosystems). The list of the primer pairs and their forward and reverse sequences are shown in Table 1.

Table 1: The forward and reverse sequences of the primer pairs used in this study

SSR Marker	Forward sequence	Reverse sequence
RM297	TCTTTGGAGGCGAGCTGAG	CGAAGGGTACATCTGCTTAG
RM7200	TCGATGGTGACGATGATACG	ACACAACAAAGGGATGGTCC
RM19106	TTTCACTTGTGAGGGATGAGTCG	GCTCCCAGCATGTTACTCTTTGG
RM1377	ATTAGATACATCAGCGGGGG	GCTGCTGTACGATGTGATCC
RM5434	GTTGATTCTGCGCGAGTTTC	GAAACCGCCCACGCAAAC
RM24545	ACAGCACAGCACCCGGAAGG	CGAGCAACAGGAAGGCGATAAGC
RM1125	GGGGCCAGAGTTTTCTTCAG	GTACGCGCAGAAAATGAGAG
RM330A	CAATGAAGTGGATCTCGGAG	CATCAATCAGCGAAGGTCC
RM7619	CTTGGTATGTATTGGCAGCG	GAGGCAATAGGAGGGGAGAG

Data analysis

The molecular data obtained were analysed to determine the direction and rate (frequency) of gene flow between *O. longistaminata* and each of the two varieties of *O. sativa*. Presence of alleles of parental strains or alleles of one of the two species into the progenies of the other species was used as an indicator of gene flow between them (Harrison and Larson 2014). The rate or frequency of gene flow between *O. longistaminata* and each of the two local varieties of *O. sativa* was determined as the proportion of the hybrid progenies to total number of progenies of a particular species or variety in the sample (Arias and Rieseberg 1994). Population assignment analysis was performed using *GenAlEx* (Version 6.41) software package in order to show clustering pattern of *O. longistaminata*, each of the two *O. sativa* varieties and their hybrids.

Results

Gene flow between *Oryza longistaminata* and *Oryza sativa*, var. zambia

Molecular data analysis using SSR markers revealed that 5.3 % of progenies of *O. sativa* var. zambia possessed some *O. longistaminata*-specific alleles, implying that they were hybrids. On the other hand, 30% of the progenies of *O. longistaminata* plant possessed *O. sativa* (var. zambia) - specific alleles, indicating that they were hybrids formed between *O. longistaminata* and *O. sativa*, var. zambia. All hybrid progenies displayed stable *O. longistaminata* (L) *O. sativa (*S) heterozygous alleles. The allelic composition of the four hybrids (H1, H2, H3 and H4) resulting from gene flow between *O. longistaminata* and *O. sativa* var. zambia rice based on the nine SSR markers is presented in Table 2.

Table 2: Schematic presentation of microsatellite DNA profiling for hybrids and their parents.

	Genotypes							
SSR Marker	**H1**		**H2**		**H3**		**H4**	
RM297								
RM330A								
RM1125								
RM1377								
RM5434								
RM7200								
RM7619								
RM19106								
RM24545								
Hybrids	**L**	**S**	**L**	**S**	**L**	**S**	**L**	**S**

Each column corresponds to a hybrid and consists of two sub-columns, L (*O. longistaminata*) and S (*O. sativa*) lines. Each row shows the allelic pattern of each SSR marker in the four hybrids. The shaded blocks correspond to the presence of different allele in respective marker.

Population assignment analysis of *Oryza longistaminata*, *Oryza sativa* (var. zambia) and their hybrids

Population assignment analysis performed based on the SSR data showed clustering of *O. longistaminata*, *O. sativa* var. zambia and their hybrids into three distinct groups or populations. The hybrids clustered together as a distinct population between the *O. longistaminata* and *O. sativa* var. zambia populations. The clustering pattern of *O. longistaminata*, *O. sativa* var. zambia and hybrid populations is shown in Figure 3.

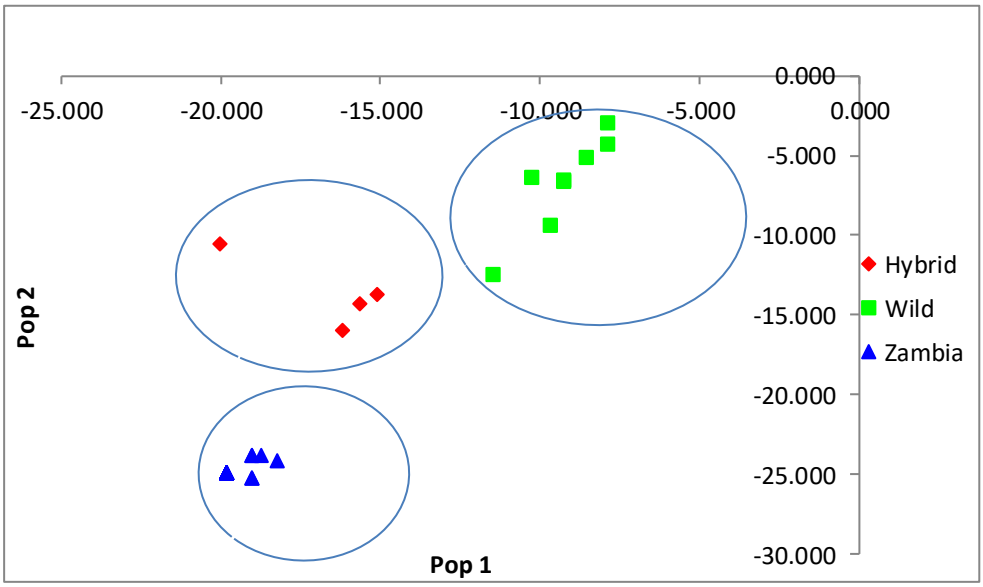

Figure 3: Clustering of *O. longistaminata*, *O. sativa* var. zambia and their hybrids based on population assignment analysis.

Gene flow between *Oryza longistaminata* and *Oryza sativa* var. kalamata

Molecular analysis using SSR markers did not detect any *O. longistaminata*-specific alleles in any of the progenies of *O. sativa* var. kalamata, implying that no gene flow had occurred from *O. longistaminata* to *O. sativa* var. kalamata. On the other hand, 27.3% of the progenies of *O. longistaminata* possessed *O. sativa* var. kalamata-specific alleles, implying that they were hybrids. The allelic composition of the three hybrid individuals (referred here to as H5, H6 and H7) that resulted from the gene flow between *O. longistaminata* and *O. sativa* var. kalamata based on the nine SSR markers are presented in Table 3.

Table 3: Schematic presentation of microsatellites DNA profiling for hybrids and their parents.

	Genotypes					
SSR Marker	**H5**		**H6**		**H7**	
RM297						
RM330A						
RM1125						
RM1377						
RM5434						
RM7200						
RM7619						
RM19106						
RM24545						
Hybrids	**L**	**S**	**L**	**S**	**L**	**S**

Each column corresponds to a hybrid and consists of two sub-columns, L (= *O. longistaminata*) and S (= *O. sativa*) lines. Each row shows the allelic pattern of each SSR marker in the three hybrid individuals. The shaded blocks correspond to the presence of different allele in respective marker.

Population assignment analysis of *Oryza longistaminata, O. Sativa* var. Kalamata and their hybrids

Population assignment analysis performed based on the microsatellites/SSR data grouped *O. longistaminata*, *O. sativa* var. kalamata and their hybrids into three distinct clusters or populations that were separate from each other (Figure 4). The hybrids clustered together (as a population) between the *O. longistaminata* and *O. sativa* var. kalamata populations.

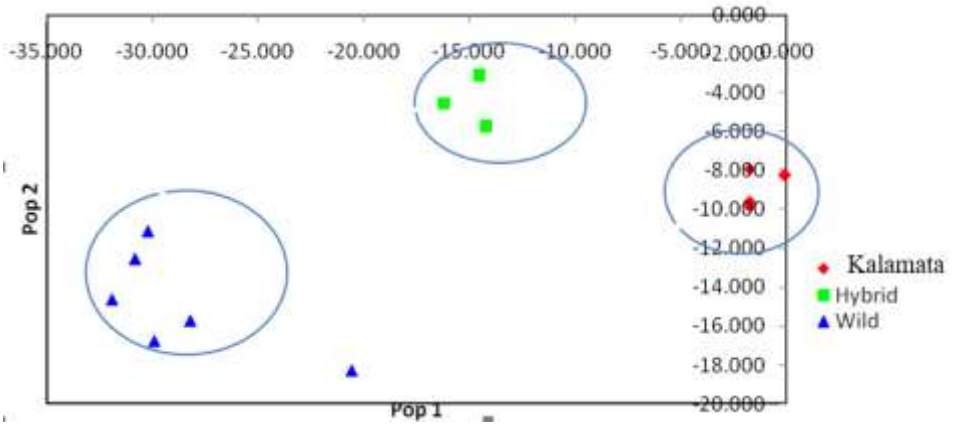

Figure 4: Clustering of *O. longistaminata*, *O. sativa* var. kalamata and their hybrids based on the population assignment analysis.

Discussion

This study evaluated the direction and extent or rate of pollen-mediated gene flow between *O. longistaminata* and two varieties of cultivated rice, *O. sativa* var. kalamata and var. zambia using SSR markers. The molecular markers (SSR markers) used in this study were found to be highly informative and suitable for rice population studies as demonstrated by high amplification levels. The high level of amplification of these markers was also reported by Mangosongo et al. (2020) in their study on genetic diversity of *O. longistaminata* populations in Tanzania. The present study revealed that gene flow between *O. longistaminata* and the two varieties of *O. sativa* occurs naturally when they grow sympatrically and when their flowering periods overlap. The implication of these results is that the two species are sexually compatible, hence they can naturally hybridize. These results seem to concur with the findings of the studies by Kiambi et al. (2008) and Kanya et al. (2012) who reported the possibility of hybridization between *O. longistaminata* and *O. sativa*. Flow of genes from the wild species (which usually possess high genetic diversity) to cultivated can help to enrich the genetic base of the cultivated species and hence improving the yield (productivity) of cultivated rice. Contrary, flow of genes from cultivated (crop) to the wild species can result into reduction in genetic variability in the wild species or may replace genes of wild species through genetic assimilation, making the wild species populations vulnerable to environmental stochasticity (Haygood et al. 2003).

The study further revealed that gene flow between the two species investigated was bi-directional. However, the major direction of gene flow was from cultivated rice species (*O. sativa*) to the wild rice species (*O. longistaminata*). These results seem to concur with what was reported by Morishima et al. (1984) who pointed out that the major direction of gene flow in *O. sativa* complex is from annual to perennial because the outcrossing rates are much higher in perennial than in annual types. Of the two species assessed in the present study *O. sativa* is an annual species while *O. longistaminata* is a perennial species. The variation in outcrossing rates between the two rice species is probably due to difference in their breeding systems (Song et al. 2003), in which the *O. sativa* is essentially a self-pollinated species while *O. longistaminata* is a cross-pollinated species (Kiambi et al. 2008, Mangosongo 2015). According to the results, the rates of gene flow from the two *O. sativa* varieties to *O. longistaminata* were 27.3% and 30% for *O. sativa* var. kalamata and *O. sativa* var. zambia respectively, while

from *O. longistaminata* to *O. sativa* var. kalamata and var. zambia were 0% and 5.3% respectively. Similar to the findings of the study by Song et al. (2003) the results of this study showed that the rate of gene flow between the wild and cultivated rice species varies with the cultivated rice variety involved. The rates of gene flow found in this study correspond or were within the range that was reported in the study by Langevin et al. (1990) on gene flow between *O. sativa* and the weedy rice, *O. sativa* f. spontanea using allozymes in which the rate of hybridization was found to range from 1% to 52%, depending on the rice cultivar acting as pollen donor. The finding of this study is also consistent withthat reported in the study by Noldin et al. (2002) who reported occurrence of bi-directional gene flow between the wild rice species, *O. punctata* and cultivated rice, *O. sativa*.

The literature further shows that variation in the rate of gene flow between plant species can be attributed by their differences in floral morphology or structure and the time their stigmas are receptive (Messeguer et al. 2001, Mangosongo 2015). The floral structure of *O. sativa*, such as short stigmas, limits the chances of outcrossing (Messeguer et al. 2001). In contrast, the floral structure of *O. longistaminata* such as long and outstretched stigmas increases the opportunity of capturing foreign pollen grains (Song et al. 2003). Therefore, the difference in floral structure between the *O. sativa* and *O. longistaminata* can be used to account for their differences in the rates of gene flow. Similarly, studies by Oka (1988) and Morishima et al. (1992) revealed that among the features that limit cross pollination in *O. sativa* include short styles and stigmas, short anthers, limited pollen viability and very short period between opening of florets and release of pollens. On the other hand, *O. longistaminata* has longer styles, stigmas andanthers and its pollen remains viable twice as long as in *O. sativa* (Kanya et al. 2012), hence increasing the chance for cross pollination.

Conclusion

This study revealed that gene flow between *O. longistaminata* and the two cultivated rice varieties, *O. sativa* var. kalamata and *O. sativa* var. zambia occurs naturally when they grow sympatrically and when they have synchronized flowering periods. The direction of gene flow is generally bi-directional, but occurs more frequently from cultivated (*O. sativa*) to wild (*O. longistaminata*) than from wild (*O. longistaminata*) to cultivated (*O. sativa*). Generally, the rate of gene flow between wild rice to cultivated rice varies with the cultivated rice variety involved. The findings of this study provide useful information for conservation, management and maintenance of genetic diversity of the wild and cultivated rice species through control of pollen-mediated gene flow between the two species.

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