PHILIPP AGRIC SCIENTIST
Vol. 104 No. 2, 146-156
June 2021

ISSN 0031-7454

Weedy Rice Conserved *Ex Situ* Characterized Using Morphological and Simple Sequence Repeat (SSR) Markers

Rubylyn Mijan-Infante[1,2,*], Maria Elizabeth B. Naredo[3], and Merlyn S. Mendioro[4]

[1]Department of Biology, College of Arts and Sciences, Batangas State University, Rizal Avenue, Batangas City 4200, Philippines.
[2]Graduate School, University of the Philippines, Los Baños, Laguna 4031, Philippines.
[3]Institute of Biology, University of the Philippines, Diliman, Quezon City 1101 Philippines.
[4]Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines, Los Baños, Laguna 4031, Philippines.

[*]Author for correspondence; E-mail: rdmijan@up.edu.ph

Received: 21 September 2020/ Revised: 01 March 2021/ Accepted: 11 March 2021

A clear understanding of genetic diversity in weedy rice is important in improving protocols for its conservation, *ex situ*. To this end, Asian collections of weedy rice together with accessions of the wild AA genome species *Oryza nivara* Sharma et Shastry and *Oryza rufipogon* Griffith, and the cultivated species, *Oryza sativa* L. were characterized using 16 qualitative morphological traits and 18 SSR markers. The Shannon-Weaver index (*H'*) revealed higher diversity in the weedy (mean *H'* = 0.76) and wild *O. nivara* (mean *H'* = 0.79) and *O. rufipogon* samples (mean *H'* = 0.69) compared to the cultivated samples (mean *H'* = 0.44). The weedy forms showed high preference (> 90%) for high grain shattering and awn presence. Cluster analysis based on the 16 qualitative characters revealed three major clusters where weedy accessions grouped with (1) *O. rufipogon* samples (2) *O. nivara*, *O. rufipogon* and indica rice samples, and (3) indica, aus, and japonica and a few *O. nivara* and *O. rufipogon* rice samples. Cluster analysis based on SSR data concurred largely with morphological analysis. Genetic diversity indices (number of alleles, observed heterozygosity, expected heterozygosity) detected by SSR markers were comparable between the weedy (1.64, 0.11, 0.23) and *O. rufipogon* (1.53, 0.19, 0.23) samples. Analysis of molecular variance (AMOVA) among the weedy forms revealed that differentiation within accessions accounted for 79% of the total variation.

Keywords: *Ex situ* conservation, genetic diversity, genebank management, germplasm regeneration, weedy rice, *Oryza* germplasm

Abbreviations: AMOVA—analysis of molecular variance, CULMHAB—culm habit, FLATT—flag leaf attitude, IRG—international rice genebank, PIC—polymorphic information content, SOP—standard operating procedures.

INTRODUCTION

Rice (*Oryza sativa* L.) is the most important cereal food providing a staple source of energy, protein, and other nutrients to more than 50% of the rapidly increasing population in Asia, Africa, and South America (Surridge 2004). It is the most demanded food crop with an expected increase of 38% within 30 years (Joseph et al. 2010). Rice production has, however, substantially slowed down in many areas due to bio-physical constraints that affect yield including water management, soil nutrient depletion, change in rainfall patterns, weeds, and pests and diseases (Musiime et al. 2005). Of these, weeds are one of the major threats to rice productivity worldwide.

Weedy rice refers to all unwanted populations of the genus *Oryza* growing in and around rice fields (Nadir et al. 2017). Its proliferation is exacerbated by the practice of direct seeding of fields (Olajumoke et al. 2015; Sudianto et al. 2016). It is one of the most dominant and competitive weed species found in rice planting areas worldwide possessing aggressive traits such as rapid growth, high tillering, enhanced ability for fertilizer uptake, asynchronous maturation, seed shattering, and long dormancy periods (Shrestha et al. 2018). Yield loss attributed to weedy rice ranges from 10% to 100% (Marambe and Amarasinghe 2000) or about 60-74% losses if it comprises 35% of the crop in the field (Watanabe et al. 1997; Marambe 2009).

Weedy rice can be a natural hybrid of cultivated rice and wild rice species. It has traits associated with its wild relatives including increased plant height and awned, shattering seeds with red pericarp (Olajumoke et al. 2015). Weedy rice in Thailand might have originated from the introgression between cultivated rice and *O. rufipogon* (Prathepha 2009). In areas where wild rice is absent,

weedy forms are probably the derivatives of cultigens e.g., arising as an off-type caused by genetic mutation accompanied by the development of a strong shattering habit (Akasaka et al. 2009). Weedy rice derived from cultivated rice does not escape from the agricultural setting and remain confined to rice fields and disturbed surroundings (Vaughan et al. 2005).

Weedy rice shows varying degree of sexual compatibility with the cultivated species and has been reported to have an out-crossing rate of 1-52% (Langevin et al. 1990). It therefore appears to be the main candidate for gene flow and introgression of traits such as higher tolerance to changes in environmental conditions, since it is compatible and usually intermingled with the rice crop (Suh 2003). Desirable agronomic traits in weedy rice have been reported including increased biomass production and high seed yields at elevated temperatures (Ziska et al. 2012; Nadir et al. 2017) and tolerance to abiotic stresses like drought, cold, and salinity and biotic pressures such as blast and bacterial diseases (Suh 2003). A rice variety, Cheonsu with drought and cold tolerance, was developed using a japonica cultivar and a weedy rice as parents (Suh 2008; Nadir et al. 2017). Because of its potential in rice improvement, genetic diversity in weedy rice should be safeguarded.

Ex situ conservation of rice germplasm is the most efficient method to safeguard diversity in rice genetic resources (Jackson 1997). For instance, the International Rice Genebank (IRG) at the International Rice Research Institute in the Philippines holds in trust more than 130,000 accessions of rice germplasm (CGIAR Genebank Platform) of which about 1,100 accessions are considered weedy (Hilario 2020, pers comm). Conservation of weedy rice is a major challenge due to its ambiguous nomenclature and taxonomic status and complex biosystematics relationships with AA genome *Oryza* species. This study aimed to characterize the genetic diversity in *ex situ* collections of weedy rice using morphological and molecular markers to further improve protocols to conserve weedy rice populations, *ex situ*.

MATERIALS AND METHODS

Plant Materials and Establishment

Thirty-one accessions of weedy rice, ten *O. nivara*, nine *O. rufipogon*, and seven *O. sativa* accessions were provided by the IRG (Table 1). The choice of origin of the materials was limited to Asia. Species designation was based on IRG classification scheme (https://www.genesys-pgr.org/a/v2RV32jqq4p). Accessions designated as *Oryza* sp., *O. nivara* x *O. sativa*, and *O. rufipogon* x *O. sativa* were considered as weedy types based on taxonomic remarks and notes from passport information (e.g., % introgression, distance from farmer's field). The identification of weedy accessions was validated based on seed file morphology and morphological characters (Hilario, pers comm). True-to-types accessions were represented by *O. nivara*, *O. rufipogon*, and *O. sativa*.

Seeds were oven dried at 50°C for seven days and dehulled to break seed dormancy. Seeds were germinated on petri dishes lined with moist filter paper at 30°C temperature and 99% relative humidity. Germinated seeds that showed vigorous radicles were established in seed boxes and transplanted singly to 15 cm diameter x 19 cm tall pots at 45 days after seeding. Each pot was considered a replication. An accession was considered a treatment with four replications arranged in a completely randomized block design. The study was conducted in Los Baños, Laguna Philippines, from April 2015 to May 2016.

Morphological Characterization and Analysis

The plants were characterized for 16 qualitative traits of the leaf, culm, panicle, and spikelet taken at appropriate growth stages (Table 2). Character states were adapted from Descriptors for Wild and Cultivated Rice (*Oryza* spp.) (Bioversity International, IRRI and WARDA 2007). The frequency distributions of each trait category were calculated. The Shannon-Weaver diversity index (*H'*) was computed using frequency distributions of each trait to assess the diversity for each character. The Shannon-Weaver diversity index was computed as:

Eq. 1 $$H' = -\sum_{i=1}^{n} p_i \log_e(p_i)$$

where p_i is the proportion of accessions in the i^{th} class of an n-class character and n is the number of phenotypic classes of traits (Ortiz-Burgos 2016). Each H' value was normalized by dividing by its maximum value ($\log_e n$) in order to keep the values between 0 and 1. Cluster analysis was carried out using DARwin 5.0 (Perrier et al. 2003) to generate a dendrogram using Unweighted Neighbor-Joining algorithm.

Molecular Characterization and Analysis

Total DNA from individual plants for each accession was isolated according to the modified protocol of Fulton et al. (1995). An accession was represented by four plants, the usual number used in the old protocol for seed multiplication and regeneration in IRG. A total of eighteen microsatellite primers previously localized in weedy rice (Cao et al. 2006; Prathepha 2011; Li et al. 2015; Recio 2015) were used in this study (Table 3). A 15 ul PCR reaction with a total of 5 ng DNA contained 1 X PCR buffer (100 mM Tris-HCl, 500 mM KCl and 0.1% Gelatin), 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.10 μM forward primer, 0.10 μM reverse primer, and 0.04 units of Taq polymerase

Table 1. *Oryza* germplasm represented by weedy and true-to-type accessions subjected to morphological and molecular characterization.

Accession Number	Origin	Species Designation	Taxonomist's Remarks/Collector's Notes
80451	India	*O. nivara x O. rufipogon*	far from *O. sativa field*
80429	India	*O. nivara x O. sativa*	2 - 5% introgression
80443	India	*O. nivara x O. sativa*	in cultivated field
80456	India	*O. nivara x O. sativa*	40 - 60% introgression
80586	India	*O. nivara x O. sativa*	field border
86573	Cambodia	*O. nivara x O. sativa*	hybrid swarm
80508	India	*O. rufipogon x O. sativa*	hybrid swarm; 20 - 40% introgression
80745	Myanmar	*O. rufipogon x O. sativa*	hybrid swarm; in cultivated field
81938	India	*O. rufipogon x O. sativa*	hybrid swarm; no introgression
86522	Vietnam	*O. rufipogon x O. sativa*	heavily introgressed
89096	Laos	*O. rufipogon x O. sativa*	hybrid swarm
89233	Cambodia	*O. rufipogon x O. sativa*	no introgression
93239	Nepal	*O. rufipogon x O. sativa*	hybrid swarm; heavily introgressed
99599	Indonesia	*O. rufipogon x O. sativa*	
103814	China	*O. rufipogon x O. sativa*	hybrid swarm
105367	Thailand	*O. rufipogon x O. sativa*	hybrid swarm; 10 - 20% introgression
106431	Vietnam	*O. rufipogon x O. sativa*	no introgression
80428	India	*Oryza sp.*	Asian weedy rice; 2 - 5% introgression
80628	India	*Oryza sp.*	Asian weedy rice; <1% introgression
81970	Thailand	*Oryza sp.*	Asian weedy rice; 20 - 40% introgression
86521	Vietnam	*Oryza sp.*	Asian weedy rice; in cultivated field
89113	Laos	*Oryza sp.*	Asian weedy rice; 40 - 60% introgression
89239	Cambodia	*Oryza sp.*	Asian weedy rice; 20 - 40% introgression
89266	Cambodia	*Oryza sp.*	Asian weedy rice; no introgression
93235	Nepal	*Oryza sp.*	Asian weedy rice; 6 - 10% introgression
100187	Malaysia	*Oryza sp.*	Asian weedy rice
101943	Myanmar	*Oryza sp.*	Asian weedy rice
104634	China	Oryza sp.	Asian weedy rice
105488	China	*Oryza sp.*	Asian weedy rice
105940	Thailand	*Oryza sp.*	Asian weedy rice; <1% introgression
106165	Laos	*Oryza sp.*	Asian weedy rice
81844	India	*O. nivara*	
86496	Vietnam	*O. nivara*	
89068	Laos	*O. nivara*	
89183	Cambodia	*O. nivara*	
93185	Nepal	*O. nivara*	
103824	China	*O. nivara*	
103836	Bangladesh	*O. nivara*	
105442	Sri Lanka	*O. nivara*	
105765	Thailand	*O. nivara*	
106397	Myanmar	*O. nivara*	
81881	India	*O. rufipogon*	
82988	China	*O. rufipogon*	
93216	Nepal	*O. rufipogon*	
99534	Cambodia	*O. rufipogon*	
105839	Thailand	*O. rufipogon*	
105899	Bangladesh	*O. rufipogon*	
105952	Indonesia	*O. rufipogon*	
106157	Laos	*O. rufipogon*	
106508	Myanmar	*O. rufipogon*	
117264	Philippines	*O. sativa*	tropical japonica
117266	India	*O. sativa*	aus
117268	Philippines	*O. sativa*	indica
117271	China	*O. sativa*	indica
117274	Japan	*O. sativa*	temperate japonica
117280	China	*O. sativa*	indica
117281	Bangladesh	*O. sativa*	indica

(Intron Biotechnologies). The amplification was run using G-Storm™ thermocycler with the following amplification profile: initial denaturation at 95°C (2 mins), 35 cycles of denaturation at 94°C (30 sec), annealing at 55°C (30 sec), and elongation at 72°C (50 sec), followed by a 3 min final elongation step at 72°C. Electrophoresis was performed on 8% polyacrylamide using the Mega-Gel High-Throughput Electrophoresis System C-DASG-500-50 (CBS).

Genetic polymorphisms for each locus were assessed by calculating the polymorphic information content (PIC), allele number (*Na*) (Hartl and Clark 1997), expected heterozygosity (*He*) (Nei 1973) and observed heterozygosity (*Ho*) using GenAlEx (Peakall and

Table 2. Qualitative morphological characters taken at specific growth stages.

Plant Part	Character	Code	Growth Stage Taken/ Description
Leaf	Basal leaf sheath color	BLSCO	Late vegetative/color of the outer surface of the leaf sheath
	Leaf blade anthocyanin color	LBACO	Late vegetative
	Leaf auricle color	AUCO	Late vegetative
	Collar color	COLLCO	Late vegetative
	Ligule color	LIGCO	Late vegetative
	Flag leaf attitude	FLATT	7 days after anthesis/ angle of attachment between the flag leaf blade and main panicle axis
	Leaf senescence	LEAFSEN	At harvest/estimated by observing greenness of leaves below the flag leaf
Culm	Node anthocyanin color	NOCO	Flowering to maturity/ presence and distribution of purple color on the outer surface of the node
	Internode anthocyanin color	INCO	Near maturity/ presence and distribution of purple color on the outer surface of the internode
	Culm habit	CULMHAB	After flowering/ estimated average angle of inclination of the base of the main culm from vertical
Spikelet	Lemma and palea color	LPCO	At anthesis
	Lemma color of apiculus	APCO	At anthesis
	Awn presence	AWNPRES	Flowering to maturity
Panicle	Panicle attitude of branches	PANATT	After anthesis/ compactness of the panicle according to its mode of branching
	Panicle exsertion	PANEXS	Near maturity/extent to which the panicle is exserted above the flag leaf sheath
	Panicle shattering	PANSHATT	At maturity or harvest/ extent to which grains have shattered from the panicles

Smouse 2006; 2012). Analysis of molecular variance (AMOVA) was computed also using GenAlEx. Cluster analysis was carried out using DARwin 5.0 (Perrier et al. 2003). The relationships between all pairs of genotypes were visualized as dendrograms.

RESULTS

Morphological Characterization of Weedy Rice Population

Morphological characterization of weedy accessions revealed a variation pattern that differed from the true-to-type *O. nivara* and *O. rufipogon* and cultivated species (Table 4). The weedy rice samples showed considerable morphological variation in leaf, culm, and panicle attributes. Green coloration was more predominant (> 50%) than purple in the leaf and culm attributes. Purple coloration was observed as the presence of anthocyanin in varying degrees (e.g., light purple and purple BLSCO) in the leaf and culm including the BLSCO (45.5%), LBACO (41.8%), AUCO (35.8%), COLLCO (41.2%), and LIGCO (37.0%), NOCO (32.7%) and INCO (26.0%). The presence of anthocyanin was predominant in *O. nivara* and *O. rufipogon* specifically on BLSCO (87.5% and 88.2%) and LBACO (83.3% and 76.5%). The cultivated accessions were generally devoid of anthocyanin occurring only at about 10% of the samples. Anthocyanin occurred less frequently on the lemma and palea (LPCO) with < 20% in the weedy and *O. nivara* rice samples and none on the *O. rufipogon* and cultivated samples. The flag leaf attitude (FLATT) that reflects the angle of attachment between the flag leaf blade and the main panicle axis was mostly erect (55.8% and 50%) or semi-erect (25.5% and 33.3%) in weedy accessions and *O. nivara* rice samples and descending flag leaf (41.2%) in *O. rufipogon*. The cultivated accessions showed only a semi-erect (80%) and horizontal (20%) FLATT. Culm habit (CULMHAB) or the estimated average angle of inclination of the base of the main culm from vertical mostly showed erect (40.0%) in the weedy samples comparable to *O. nivara* and *O. rufipogon* rice samples with open (50% and 41.2%) CULMHAB. The cultivated samples predominantly (95%) showed semi-erect CULMHAB. The mode of branching and angle of the primary branches (PANATT) was mostly compact in the weedy (51.3%) and *O. nivara* (62.5%) samples, open in *O. rufipogon* (80%) and mostly semi-compact in the cultivated species (52.6%). Horizontal and drooping panicle types were observed at very low frequencies and only in the weedy (< 6%) and cultivated samples (< 11%). The weedy and *O. nivara* accessions showed variability in the extent to which the panicle is exserted above the flag leaf sheath (PANEXS) while 90% of *O. rupifogon* and 55% of *O. sativa* showed well exserted and moderately PANEXS, respectively. Awns (AWNPRES) were present with > 90% of the weedy, *O. nivara*, and *O. rufipogon* samples but was observed only in 25% of the cultivated samples. A high percentage (84%) of the weedy samples demonstrated very high panicle shattering (PANSHATT) also observed to a lesser degree (< 50%) in *O. nivara* and *O. rufipogon* samples. The cultivated species were mostly of non-shattering types with only 5% showing high shattering. Senescence (LEAFSEN), estimated by observing all leaves below the flag leaf for their retention of greenness was recorded mostly to be late (27.7%) to very late (57.2%) in the weedy samples in contrast to the cultivated samples that

Table 3. Primer information for loci included in the molecular analysis of weedy and true-to-type *Oryza* samples.

Locus Name	Chromosome	Repeat Type	Forward Primer	Reverse Primer	Reference
RM11	7	(GA)17	tctcctcttcccccgatc	atagcgggcgaggcttag	Cao et al. (2006)
RM44	8	(GA)16	acgggcaatccgaacaacc	tcgggaaaacctaccctacc	Cao et al. (2006)
RM110	2	(GA)15	tcgaagccatccaccaacgaag	tccgtacgccgacgaggtcgag	Cao et al. (2006)
RM167	11	(GA)16	gatccagcgtgaggaacacgt	agtccgaccacaaggtgcgttgtc	Prathepa (2011)
RM216	10	(CT)18	gcatggccgatggtaaag	tgtataaaaccacacggcca	Recio (2015)
RM223	8	(CT)25	gagtgagcttgggctgaaac	gaaggcaagtcttggcactg	Recio (2015)
RM224	11	(AAG)8(AG)13	atcgatcgatcttcacgagg	tgctataaaaggcattcggg	Recio (2015)
RM230	8	(AGG)4(GA)9A(AG)13	gccagaccgtggatgttc	caccgcagtcacttttcaag	Cao et al. (2006)
RM234	7	(CT)25	acagtatccaaggccctgg	cacgtgagacaaagacggag	Recio (2015)
RM235	12	(CT)24	agaagctagggctaacgaac	tcacctggtcagcctctttc	Recio (2015)
RM248	7	(CT)25	tccttgtgaaatctggtccc	gtagcctagcatggtgcatg	Recio (2015)
RM251	3	(CT)29	gaatggcaatggcgctag	atgcggttcaagattcgatc	Recio (2015)
RM253	6	(CT)25	tccttcaagagtgcaaaacc	gcattgtcatgtcgaagcc	Cao et al. (2006)
RM257	9	(CT)24	cagttccgagcaagagtactc	ggatcggacgtggcatatg	Recio (2015)
RM276	6	(AG)8A3(GA)33	ctcaacgttgacacctcgtg	tcctccatcgagcagtatca	Cao et al. (2006)
RM277	12	(GA)11	cggtcaaatcatcacctgac	caaggcttgcaagggaag	Cao et al. (2006)
RM280	4	(GA)116	acacgatccactttgcgc	tgtgtcttgagcagccagg	Recio (2015)
RM332	11	(CTT)5-12-(CTT)14	gcgaaggcgaaggtgaag	catgagtgatctcactcaccc	Recio (2015)

senesced early (42.9%) or very early (19.0%). True-to-type *O. nivara* and *O. rufipogon* samples demonstrated very early to very late LEAFSEN at comparable frequencies.

The Shannon-Weaver (H') indices that provide information on the distribution of observations for the binary or multi-state characters are presented on Table 5. A scale of diversity indices was adapted from Rabara et al. (2014) to categorize the computed indices into maximum (H' = 1.00), high (H' = 0.76 – 0.99), moderate (H' = 0.46 – 0.75) and low diversity (0.01 – 0.45). Mean standardized H' in the weedy samples (0.76) was comparable to that in *O. nivara* (0.78) and *O. rufipogon*

Table 4. Distribution of character states for qualitative characters in weedy, wild, and cultivated samples.

Character	Character State	Frequency (%)			
		Weedy	*O. nivara*	*O. rufipogon*	Cultivated
Basal leafsheath color (BLSCO)	Green	54.50	12.50	11.80	90.00
	Light purple	40.00	66.70	64.70	10.00
	Purple	5.50	20.80	23.50	0.00
Leaf blade anthocyanin color (LBACO)	Absent	58.20	16.70	23.50	100.00
	Present	41.80	83.30	76.50	0.00
Leaf auricle color (AUCO)	No anthocyanin	63.00	30.40	47.10	80.00
	With anthocyanin	35.80	69.60	52.90	15.00
Collar color (COLLCO)	No anthocyanin	58.80	29.20	52.90	85.00
	With anthocyanin	41.20	70.80	47.10	15.00
Ligule color (LIGCO)	No anthocyanin	63.00	29.20	52.90	90.00
	With anthocyanin	37.00	70.80	47.10	10.00
Culm node anthocyanin color (NOCO)	Green	67.30	33.30	52.90	90.00
	Purple	7.30	20.80	17.60	10.00
	Light purple	13.30	29.20	5.90	0.00
	Purple lines	12.10	16.70	23.50	0.00
Culm internode anthocyanin color (INCO)	Green	73.90	37.50	52.90	90.00
	Purple	4.80	37.50	23.50	5.00
	Light purple	3.00	4.20	0.00	0.00
	Purple lines	18.20	20.80	23.50	5.00
Lemma and palea color (LPCO)	No anthocyanin	82.10	83.30	100.00	100.00
	With anthocyanin	17.90	16.70	0.00	0.00
Lemma color of apiculus (APCO)	Absent	37.20	16.70	70.00	60.00
	Very weak	5.80	8.30	20.00	10.00
	Weak	34.00	37.50	0.00	20.00
	Medium	18.60	33.30	10.00	5.00
	Strong	4.50	4.20	0.00	5.00
Flag leaf attitude (FLATT)	Erect	55.80	50.00	23.50	0.00
	Semi-erect	25.50	33.30	29.40	80.00
	Horizontal	12.10	16.70	5.90	20.00
	Descending	6.70	0.00	41.20	0.00

Table 4. Continuation.

Character	Character State	Frequency (%)			
		Weedy	*O. nivara*	*O. rufipogon*	Cultivated
	Erect	40.00	16.70	0.00	0.00
	Semi-erect	28.50	16.70	23.50	95.00
Culm habit (CULMHAB)	Open	26.70	50.00	41.20	5.00
	Spreading	4.80	16.70	35.30	0.00
	Procumbent	0.00	0.00	0.00	0.00
	Compact	51.30	62.50	10.00	15.80
	Semi-compact	19.90	20.80	10.00	52.60
Panicle attitude of branches (PANATT)	Open	20.50	16.70	80.00	10.50
	Horizontal	5.80	0.00	0.00	10.50
	Drooping	2.60	0.00	0.00	10.50
	Enclosed	7.20	8.30	0.00	0.00
	Partly exserted	30.70	16.70	10.00	25.00
Panicle exsertion (PANEXS)	Just exserted	11.80	20.80	0.00	10.00
	Moderately exserted	27.50	16.70	0.00	55.00
	Well exserted	22.90	37.50	90.00	10.00
Awn presence (AWNPRES)	Absent	7.70	8.30	10.00	75.00
	Present	92.30	91.70	90.00	25.00
	Very low (<1%)	0.00	20.80	30.00	95.00
	Low (~3%)	3.20	12.50	0.00	0.00
Panicle shattering (PANSHATT)	Moderate (~15%)	9.00	12.50	20.00	0.00
	High (~35%)	9.00	8.30	20.00	5.00
	Very high (>50%)	84.00	45.80	30.00	0.00
	Very early	1.70	20.80	11.80	19.00
	Early	2.90	20.80	29.40	42.90
Leaf senescence (LEAFSEN)	Intermediate	10.40	16.70	23.50	9.50
	Late	27.70	12.50	23.50	14.30
	Very late	57.20	29.20	11.80	14.30

(0.69). High H' (> 0.78) values in the weedy and *O. nivara* and *O. rufipogon* samples for BLSCO, AUCO, COLLCO, and LIGCO reflect the relative evenness of the distribution of samples for the absence or presence of anthocyanin in leaf attributes. A low mean H' value for AWNPRES (0.42) indicate the predominance of awn presence in the weedy, *O. nivara*, and *O. rufipogon* samples. H' for seed shattering in weedy rice was low at 0.43 reflecting the predominance of very high shattering phenotypes. A low mean H' (0.44) value was observed in the cultivated species.

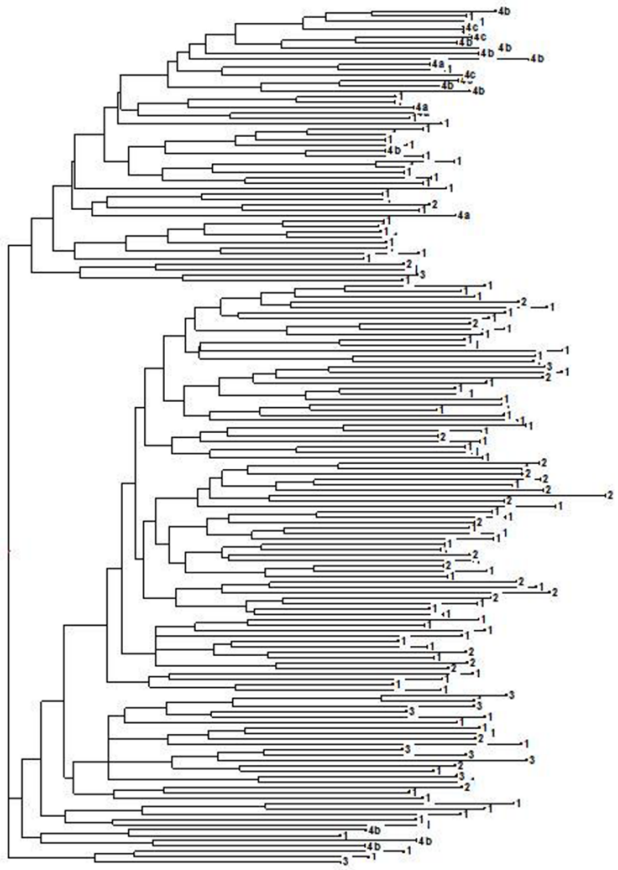
Table 5. Shannon-Weaver indices for qualitative traits in weedy, wild, and cultivated *Oryza*.

Character Code	Weedy	*O. nivara*	*O. rufipogon*	Cultivated
BLSCO	0.78	0.78	0.80	0.30
LBACO	0.98	0.65	0.79	0.00
AUCO	0.95	0.89	1.00	0.67
COLLCO	0.98	0.87	1.00	0.61
LIGCO	0.95	0.87	1.00	0.47
NOCO	0.71	0.98	0.83	0.23
INCO	0.57	0.86	0.73	0.28
LPCO	0.68	0.65	0.00	0.00
APCO	0.84	0.85	0.50	0.72
FLATT	0.80	0.73	0.89	0.36
CULMHAB	0.76	0.77	0.67	0.12
PANATT	0.77	0.57	0.40	0.83
PANEXS	0.93	0.93	0.20	0.71
AWNPRES	0.39	0.41	0.47	0.81
PANSHATT	0.43	0.88	0.85	0.09
LEAFSEN	0.67	0.98	0.96	0.91
Mean	0.76	0.79	0.69	0.44

Cluster analysis using the qualitative traits grouped the *Oryza* accessions into two major clusters (Fig. 1). In the first cluster, the weedy accessions grouped with the indica, aus, and japonica *O. sativa* samples with a lone sample of *O. rufipogon*. The second major cluster was composed of weedy, *O. nivara* and *O. rufipogon* samples. Two minor clusters were observed – one with weedy and aus *O. sativa* samples and another with weedy and *O. rufipogon* samples.

Molecular Characterization of Weedy Rice Population

All loci were polymorphic across the species with polymorphic information content (PIC) ranging from 0.80 in RM 167 to 0.97 in RM 216. The percentage polymorphic loci were comparable in *O. rufipogon* (46.9%), *O. sativa* (52.8%), and the weedy types (49.1%) and lowest in *O. nivara* (19.44%).



Legend: 1 = weedy; 2 = *O. nivara*; 3 = *O. rufipogon*; 4a = *O. sativa* indica; 4b = *O. sativa* aus; 4c = *O. sativa* temperate japonica; 4d = *O. sativa* tropical japonica

Fig. 1. Unweighted neighbor-joining dendrogram showing two major clusters based on 16 qualitative characters.

Genetic diversity parameters including allele number (*Na*), expected heterozygosity (*He*), an observed heterozygosity (*Ho*) were obtained for the Asian weedy rice and true-to-type *Oryza* species (Table 6). The weedy samples with mean *Na, Ho, He* of 1.64, 0.11, 0.23, respectively showed diversity comparable to *O. rufipogon* (1.53, 0.19, 0.23) and were more diverse than *O. nivara* (1.16, 0.04, 0.09). The *O. sativa* samples showed high mean values for *Na* (1.68), *He* (0.26) but low value for *Ho* (0.03). AMOVA results (Fig. 2) showed that 79% of the total variation is attributed to differentiation within accessions, 12% to variation within individuals, and 9% to variation among populations.

An unweighted neighbor joining tree was generated from a dissimilarity matrix of simple matching coefficients based on 18 SSR markers. The samples in Figure 3 are identified according to their status as being weedy or true-to-type wild AA genome species - *O. nivara* or *O. rufipogon*. The cultivated species, *O. sativa* was further classified according to its varietal grouping – temperate japonica (temp), tropical japonica (trop), indica

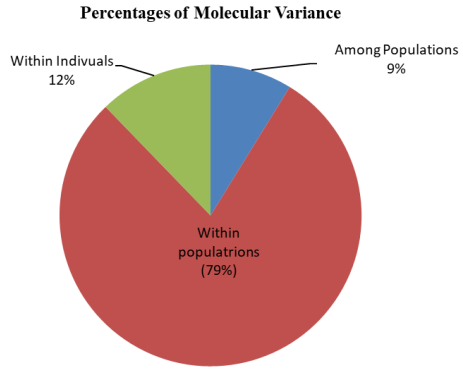


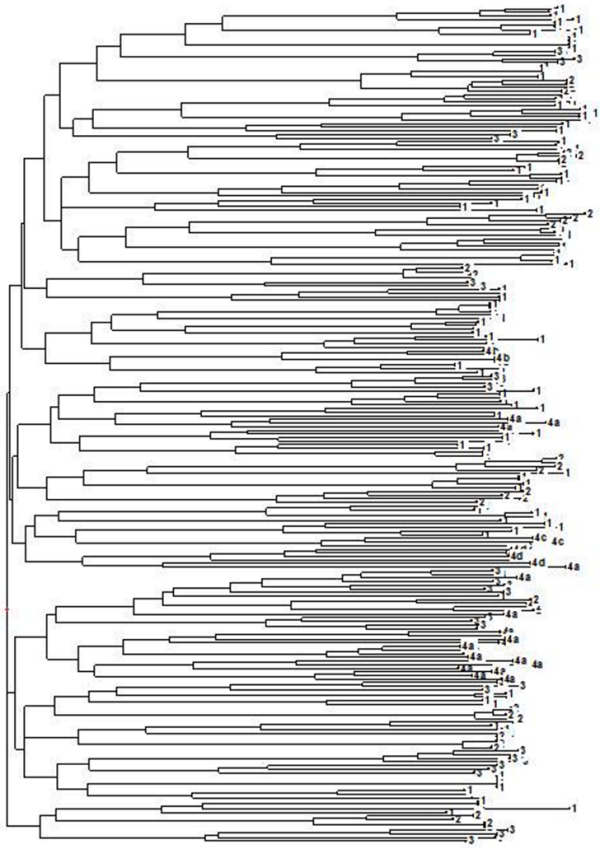
Fig. 2. Distribution of variation in weedy populations generated from Analysis of Molecular Variance using 18 SSR markers.

(ind), or aus varieties. Four major clusters were recognized. In the first cluster, weedy accessions grouped with samples of *O. nivara*, *O. rufipogon* and *O. sativa* - aus variety. The *O. sativa* - temperate and tropical japonicas, *O. nivara* and weedy samples comprised the second cluster. Most of the *O. rufipogon*, *O. nivara*, and *O. sativa* – indica samples were found in the third cluster with a few weedy accessions. The fourth cluster was composed of a weedy accession with *O. nivara* and *O. rufipogon* samples.

DISCUSSION

Awning and panicle shattering are traits attributed to weedy rice. However, the extremely high occurrence of these phenotypes in the *ex situ* weedy collections can signify loss of diversity because non-shattering forms are known to occur frequently in natural populations from Malaysia (Sudianto et al. 2016) and Sri Lanka (Perera et al. 2010) and awning is quite variable in Sri Lankan weedy rice populations (Ratnasekera et al. 2014). The genetic diversity indices detected by SSR markers were generally lower compared to that observed in natural populations of weedy and wild rice (Cao et al. 2006; Liu et al. 2015; Orn et al. 2015; Sandamal et al. 2018; Singh et al. 2018). Interestingly, the low genetic diversity in the cultivated accessions was comparable to *ex situ* collections of Philippine traditional rice varieties (Rabara et al. 2014). AMOVA results point to a larger part of the total variation accounted for by differences within accessions, reflecting the current practice of curating weedy rice as distinct populations to maximize genetic diversity at the population level.

The tendency of *ex situ* collections to become genetically diverged from their source populations is a known risk in *ex situ* conservation. Limitations in space and resources put a constraint to the number of seeds that can be used in the initial and subsequent seed



Legend: 1 = weedy; 2 = *O. nivara*; 3 = *O. rufipogon*; 4a = *O. sativa* indica; 4b = *O. sativa* aus; 4c = *O. sativa* temperate japonica; 4d = *O. sativa* tropical japonica

Fig. 3. Unweighted neighbor-joining dendrogram showing two major clusters based on 16 qualitative characters.

multiplications. The variation and genetic diversity patterns of *ex situ* collections of weedy rice observed in the present study reinforce the need for the implementation and upgrading of guidelines during regeneration, considered by Rao et al. (2006) as the most critical operation in genebank management. Regeneration should be limited to ensuring seed availability, avoiding excessive regeneration cycles. Loss of alleles due to genetic drift can be circumvented by using more parents for regeneration, although this should be balanced with resource and logistic limitations (Sackville Hamilton and Chorlton 1997; Griffith et al. 2017). Sarmiento (2017) suggested that more than 50 plants are needed to capture, with 70% probability, at least one copy of alleles with rare frequencies. In IRG, up to 50 plants are currently being used during regeneration as a compliance to standard operating procedures (SOPs) for wild and weedy rice to ensure that maximum genetic diversity is maintained during conservation.

Table 6. Measures of diversity in weedy, wild, and cultivated *Oryza* accessions.

Species	Accession Number	Allele Number (*Na*)	Observed Heterozygosity (*Ho*)	Expected Heterozygosity (*He*)
Weedy	80428	1.50	0.14	0.18
	80429	1.11	0.00	0.05
	80443	1.06	0.06	0.03
	80451	1.28	0.06	0.11
	80456	1.50	0.15	0.20
	80508	2.11	0.29	0.35
	80586	1.06	0.06	0.06
	80628	1.61	0.07	0.24
	80745	2.33	0.20	0.39
	81938	1.39	0.07	0.18
	81970	1.50	0.08	0.19
	86521	1.67	0.04	0.21
	86522	2.06	0.37	0.37
	86573	1.56	0.14	0.22
	89096	1.44	0.13	0.16
	89113	1.83	0.06	0.29
	89233	1.50	0.05	0.19
	89239	1.83	0.10	0.26
	89266	2.06	0.13	0.37
	93235	1.17	0.00	0.06
	93239	2.39	0.11	0.41
	99599	1.67	0.15	0.24
	100187	1.33	0.07	0.17
	101943	1.11	0.00	0.07
	103814	3.11	0.26	0.54
	104634	1.28	0.08	0.12
	105367	1.94	0.19	0.33
	105488	0.94	0.06	0.03
	105940	1.56	0.06	0.23
	106165	2.17	0.18	0.36
	106431	1.89	0.07	0.39
Mean		1.64	0.11	0.23
O. nivara	81844	1.00	0.00	0.02
	86496	1.00	0.00	0.05
	89068	1.28	0.01	0.11
	89181	1.11	0.00	0.05
	93185	1.17	0.00	0.07
	103824	1.28	0.14	0.11
	103836	0.89	0.00	0.00
	105442	1.06	0.06	0.15
	105765	1.11	0.01	0.07
	106397	1.72	0.14	0.25
Mean		1.16	0.04	0.09
O. rufipogon	81881	1.28	0.10	0.13
	82988	1.89	0.26	0.30
	93216	1.33	0.04	0.11
	99534	1.39	0.17	0.23
	105839	1.72	0.33	0.29
	105899	1.44	0.11	0.19
	105952	1.61	0.22	0.28
	106157	1.67	0.31	0.31
	106508	1.44	0.18	0.23
Mean		1.53	0.19	0.23
O. sativa	117266	1.22	0.01	0.17
	117268	2.22	0.04	0.39
	117271	1.94	0.01	0.34
	117280	1.00	0.04	0.05
	117281	1.44	0.04	0.21
	117274	1.94	0.04	0.34
	117264	2.00	0.03	0.34
Mean		1.68	0.03	0.26

The affinity of weedy rice to *O. nivara* and *O. rufipogon* and cultivated species demonstrated by cluster analysis based on morphological and molecular markers impact issues of containment and genetic contamination during regeneration. Wild *Oryza* including weedy accessions are to be considered as invasive species and should be grown under contained facilities. Cross contamination is managed in some genebanks (e.g., IRG) by panicle bagging or isolation through distance and physical barriers and ensuring that no plants of cultivated species are planted in the vicinity (IRRI 2018). With *a priori* knowledge of flowering time of the accessions, a staggered planting regime, timed to preclude simultaneous flowering is also recommended.

CONCLUSION

The results showed the genetic diversity and variation patterns of weedy rice conserved *ex situ*. Possible loss of diversity for both weedy and wild rice is suggested both by morphological and molecular analyses. Cluster analysis showed the affinity of the weedy samples to both wild and cultivated forms. The results reinforce the need to establish guidelines specifically during germplasm regeneration to address risks associated with loss of diversity and genetic contamination.

REFERENCES CITED

AKASAKA M, USHIKI J, IWATA H, ISHIKAWA R, ISHII T. 2009. Genetic relationships and diversity of weedy rice (*Oryza sativa* L.) and cultivated rice varieties in Okayama Prefecture, Japan. Breed. Sci. 59: 401-409.

BIOVERSITY INTERNATIONAL, IRRI, WARDA. 2007. Descriptors for wild and cultivated rice (*Oryza* spp.). Bioversity International, Rome, Italy; International Rice Research Institute, Los Baños, Philippines; WARDA, Africa Rice Center, Cotonou, Benin.

CAO Q, LU B-R, XIA H, RONG J, SALA F, SPADA A, GRASSI F. 2006. Genetic diversity and origin of weedy rice (*Oryza sativa* f. *spontanea*) populations found in North-eastern China revealed by simple sequence repeat (SSR) Markers. Annals of Botany 98: 1241–1252.

CGIAR Genebank platform. https://www.genebanks.org/genebanks/irri/.

FULTON TM, CHUNWONGSE J, TANKSLEY SD. 1995. Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Mol Biol Rep 13, 207–209.

GRIFFITH MP, CALONJE M, MEEROW AW, FRANCISCO-ORTEGA J, KNOWLES L, AGUILAR R, TUT F, SÁNCHEZ V, MEYER A, NOBLICK LR, MAGELLAN TM. 2017. Will the same *ex situ* protocols give similar results for closely related species? Biodivers Conserv 26: 2951–2966. DOI 10.1007/s10531-017-1400-2.

HARTL DL, CLARK AG. 1997. Principles of population genetics. 3rd edn. Sinauer Associates, Inc. Publishers; Sunderland, MA, USA.

[IRRI] INTERNATIONAL RICE RESEARCH INSTITUTE. 2018. Seed regeneration and morphological characterization of wild *Oryza* species (IRRI-SOP-REG and CHA Wild-1.0). Contributors: Maria Celeste. Banaticla-Hilario, Janice Victoria, Flora De Guzman, Stephen Timple, Grace Lee Capilit, Lilibeth Sison, Carlos Huelma and Nigel Ruaraidh Sackville Hamilton.

JACKSON MT. 1997. Conservation of rice genetic resources: the role of the International Rice Genebank at IRRI. Plant Mol. Biol. 35: 61-67.

JOSEPH B, JINI D, SUJATHA S. 2010. Biological and physiological perspectives of specificity in abiotic salt stress response from various rice plants. Asian J. Agric. Sci. 2: 99–105.

LANGEVIN SA, CLAY K, GRACE JB. 1990. The incidence and effects of hybridization between cultivated rice and its related weed red rice (*Oryza sativa* L). Evolution 44: 1000-1009.

LI MB, WANG H, CAO L. 2015. Evaluation of population structure, genetic diversity and origin of Northeast Asia weedy rice based on simple sequence repeat (SSR) Markers. Rice Science 22(4): 180-188.

LIU W, SHAHID MQ, BAI L, LU Z, CHEN Y, JIANG L, et al. 2015. Evaluation of genetic diversity and development of a core collection of wild rice (*Oryza rufipogon* Griff.) Populations in China. PLoS ONE10 (12):e0145990. doi:10.1371/journal. pone.0145990.

MARAMBE B. 2009. Weedy rice evolution, threats and management. Tropical Agriculturist.

MARAMBE B, AMARASINGHE L. 2000. Weedy rice in Sri Lanka. In wild and weedy rice in rice ecosystems in Asia – A Review (Eds: B.B. Baki, D.V. Chin and A.M. Mortimer). Limited Proceedings No 2. International Rice Research Institute, Philippines.

MUSIIME O, TENYWA MM, MAJALIWA MJG, LUFAFA A, NANFUMBA D, WASIGE J, WOOMER PL, KYONDHA M. 2005. Constraints to rice production in Bugiri district. African Crop Science Conference Proceedings, Vol. 7: 1495-1499.

NADIR S, XIONG H-B, ZHU Q, ZHANG X-L, XU H-Y, LI J, DONGCHEN W, HENRY D, GUO X-Q, KHAN S, SUH H-S, LEE DS, CHEN L-J. 2017. Weedy rice in

sustainable rice production. A Review. Agron. Sustain. Dev. 37: 46. DOI 10.1007/s13593-017-0456-4.

NEI M. 1973. Analysis of gene diversity in subdivided populations. Proc Natl Acad Sci USA. 70:3321–3323.

OLAJUMOKE B, JURAIMI AS, UDDIN MK, HUSNI MHA, and ALAM MA. 2015. Competitive ability of cultivated rice against weedy rice biotypes – A review. Chilean Journal of Agricultural Research. 76 (2):243-252. doi:10.4067/S0718-58392016000200015.

ORN C, SHISHIDO R, AKIMOTO M, ISHIKAWA R, HTUN TM, NONOMURA K-I, KOIDE Y, SAROM M, VANG S, SOPHANY S, MAKARA O and ISHII T. 2015. Evaluation of genetic variation among wild rice populations in Cambodia. Breeding Science 65: 430–437. doi:10.1270/jsbbs.65.430.

ORTIZ-BURGOS S. 2016. Shannon-weaver diversity index. In: Kennish M.J. (eds) Encyclopedia of Estuaries. Encyclopedia of Earth Sciences Series. Springer, Dordrecht.

PEAKALL R, SMOUSE PE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes. 6, 288-295.

PEAKALL R, SMOUSE PE. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics 28, 2537-2539.

PERERA UIP, RATNASEKERA WADPR, SENANAYAKE SGJN. 2010. Proceedings of the 15th international forestry and environment symposium, 26-27 November 2010. Published by Department of Forestry and Environmental Science, University of Sri Jayewardenepura, Sri Lanka.

PERRIER X, FLORI A, BONNOT F. 2003. Data analysis methods. In: Hamon, P., Seguin, M., Perrier, X., Glaszmann, J. C. Ed., Genetic diversity of cultivated tropical plants. Enfield, Science Publishers. Montpellier. pp 43 - 76.

PRATHEPHA P. 2009. Seed morphological traits and genotypic diversity of weedy rice (*Oryza sativa f. spontanea*) populations found in the Thai Hom Mali rice fields of north-eastern Thailand. Weed Biology and Management 9: 1-9.

PRATHEPHA P. 2011. Microsatellite analysis of weedy rice (*Oryza sativa f. spontanea*) from Thailand and Lao PDR. Aust J Crop Sci. 5: 49-54.

RABARA RC, FERRER MC, DIAZ CL, NEWINGHAM MCV, ROMERO GO. 2014. Phenotypic diversity of farmers' traditional rice varieties in the Philippines. Agronomy 4:217-241. ; doi:10.3390/agronomy4020217.

RAO NK, HANSON J, DULLOO ME, GHOSH K, NOWELL D, LARINDE M. 2006. Manual of seed handling in genebanks. Handbooks for Genebanks 8. Rome, Italy: Bioversity International.

RATNASEKERA D, PERERA UIP, HE Z. 2014. High level of variation among Sri Lankan weedy rice populations, as estimated by morphological characterization. Weed Biology and Management. 14 (1):68-75. DOI: 10.1111/wbm.12034.

RECIO JP. 2015. Genetic diversity and population structure analysis of *Oryza* AA genome species in Bago, Myanmar based on microsatellite markers. [BS thesis]. University of the Philippines. (Available at the UP Library).

SACKVILLE HAMILTON NR, CHORLTON KH. 1997. Regeneration of accessions in seed collections: a decision guide. Handbook for Genebanks No. 5. International Plant Genetic Resources Institute, Rome, Italy.

SANDAMAL S, TENNAKOON A, MENG Q-L, MARAMBE B, RATNASEKERA D, MELO A, SONG G. 2018. Population genetics and evolutionary history of the wild rice species *Oryza rufipogon* and *O. nivara* in Sri Lanka. Ecology and Evolution. 8:12056–12065. DOI: 10.1002/ece3.4665

SARMIENTO RB. 2017. Characterization of AA genome *Oryza* accessions using morphological and SSR markers to estimate optimal sample size in germplasm regeneration. [BS thesis]. University of the Philippines. (Available at the UP Library).

SHRESTHA S, STALLWORTH S, TSENG TM. 2018. Weedy rice: competitive ability, evolution, and diversity. Integrated view of population genetics, Rafael Trindade Maia and Magnólia de Araújo Campos, IntechOpen.

SINGH B, SINGH N, MISHRA S, TRIPATHI K, SINGH BP, RAI V, SINGH AK, SINGH NK. 2018. Morphological and molecular data reveal three distinct populations of Indian wild rice *Oryza rufipogon* Griff. Species Complex. Front. Plant Sci. 9: 123. doi: 10.3389/fpls.2018.00123.

SUDIANTO E, NEIK T, TAM S, CHUA T, IDRIS A, OLSEN K, SONG B. 2016. Morphology of Malaysian weedy rice (*Oryza sativa*): Diversity, origin and implications for weed management. Weed Science, 64 (3): 501-512. doi:10.1614/WS-D-15-00168.1.

SUH HS. 2003. Characterization of weedy rice germplasm. Wild crop germplasm bank, Yeungnam University, pp 1007.

SUH HS. 2008. Weedy rice. Wild Crop Germplasm Bank, Yeungnam University, pp 240.

SURRIDGE C. 2004. Rice cultivation: feast or famine? Nature 428: 360–361.

VAUGHAN DA, SANCHEZ PL, USHIKI J, KAGO A, TOMOOKA N. 2005. Asian rice and weedy rice evolutionary perspectives. Chapter 16. In crop ferality and volunteerism. Boca Raton, USA, CRC Press. 504 pp.

WATANABE KH, AZMI M, ZUKI MD. 1997. Emergence of major weeds and their population change in wet-seeded rice fields in the Muda area, Peninsular Malaysia. Proceedings of the 16th Asian Pacific Weed Science Society.

ZISKA LH, GEALY DR, TOMECEK MB, JACKSON AK, BLACK HL. 2012. Recent and projected increases in atmospheric CO_2 concentration can enhance gene flow between wild and genetically altered rice (*Oryza sativa*). PLoS One 7(5):e37522. https://doi.org/10.1371/journal.pone.0037522 https://www.genesys-pgr.org/a/v2RV32jqq4p.