

Genetic diversity and herbicide resistance of 15 *Echinochloa crus-galli* populations to quinclorac in Mekong Delta of Vietnam and Arkansas of United States

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Abstract Barnyardgrass (*Echinochloa crus-galli*) is one of the worst weeds in rice (*Oryza sativa*), but there are few reports about the genetic diversity and herbicide resistance of barnyardgrass in Vietnam. In this study, we used random amplified polymorphic DNA (RAPD) analysis and greenhouse testing to study the genetic diversity and quinclorac resistance levels of 15 *Echinochloa crus-galli* populations in the Mekong Delta, Vietnam, and the state of Arkansas, U.S. The quinclorac resistance of *Echinochloa crus-galli* populations in Vietnam was confirmed; 9 populations were resistant to quinclorac with R/S ratios ranging from 1.9 to 6.3. Six oligonucleotide primers produced a total of 55 repeatable bands of which 46 were polymorphic (83.3% average) among the 15 populations. Genetic distance was calculated, and cluster analysis separated the 15 populations into 2 main clusters with the genetic distances within the clusters ranging from 0.09 to 0.39. The two main clusters were divided into 7 subclusters, and the quinclorac resistant and susceptible populations were located randomly within each subcluster. Six out of 13 weed populations from Vietnam belonged to one cluster and a single *Echinochloa* species. The remaining 7 populations were identified as potentially different species in the *Echinochloa* genus. Nine *Echinochloa* populations from Vietnam were tested and identified as

quinclorac resistant. The connection between quinclorac resistance levels and weed groups defined by RAPD analysis in the study is unclear; the quinclorac resistance of each resistant population could have evolved individually, regardless of differences in genetic diversity and location of the sampled populations.

Keywords *Echinochloa*, RAPD, Quinclorac, Herbicide Resistance, Rice

Introduction

The barnyardgrass (*Echinochloa crus-galli*) is one of the most noxious weeds in modern agriculture because of high seed producing capability of this weed, one plant can produce 2000 to 4000 seeds in ideal condition (Gibson et al. 2002). There were about 400 weed species reported in upland crop and rice field of Vietnam, among those weeds, barnyardgrass (*Echinochloa crus-galli*) is one of the most important grass weed in rice fields, this weed can reduce approximate 25% rice yield under high infestation condition (Chin 2001). The weed is highly adapted to a wider range of photoperiods, and due to the robust morphological variations among species, the classification of *Echinochloa* remained a serious problem for weed scientists (Damalas et al. 2008). As an initial step in studying barnyardgrass in Vietnam, we used random amplified polymorphic DNA (RAPD) analysis to investigate the genetic relationship between *Echinochloa crus-galli* populations in the Mekong Delta, Vietnam. Additionally, two weed populations from the U.S. were included to determine the impact of geographical distance on genetic differences of barnyardgrass species. The LD₉₀ (lethal dose) for quinclorac was also calculated for the 15 populations in order to evaluate any

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correlation between genetic distance and herbicide resistance among weed populations.

Previously, RAPD markers were seen as an effective tool in assessing the genetic diversity of barnyardgrass in the U.S (Rutledge et al. 2000), Korea (Kim et al. 2005) and Turkey (Kaya & Menan 2011). Also, according to Marshall and Fitch, RAPD analysis could distinguish between species in the genus *Echinochloa* while both morphological and isozyme markers could not provide reliable results (Marshall & Fitch 1997). Therefore, studying genetic variation between barnyardgrass populations would give more insights about *Echinochloa* population structures in Vietnam. In 1999, the first case of quinclorac resistance in barnyardgrass was confirmed in Arkansas, U.S (Lovelace et al. 2007). In Vietnam, quinclorac has been long used to control barnyardgrass, however, before this study, quinclorac resistance in barnyardgrass had not been confirmed in Vietnam.

Materials and Methods

Plant material

Seeds from 15 *Echinochloa crus-galli* populations were randomly collected from different locations in Vietnam (13) and the U.S. (2), seed collection criteria described as below.

Source of seeds from Vietnam

During March to May 2015, using the plant phenology described by Caton et al. 2010, total 25 *Echinochloa crus-galli* seed samples were collected from rice fields in Tien Giang, Vinh Long, Can Tho, Hau Giang and Kien Giang provinces in the Mekong Delta, Vietnam, minimum distance from each location was 10 km to ensure the diversity of sampled seed, using the methods of Llewellyn et al. (2001) to collect seed, in each location, total 40–50 florets of different single plant in the field were sampled. Seeds were only collected when there were more than 20 plants bearing seeds within an area of 5000 m². Seeds were dried at room temperature for one month before planted. After planted, 13 quality samples (Fig. 2) showed consistent results in the herbicide screening test were selected for RAPD analysis.

Source of seeds from the USA

For comparison, 2 seed samples identified as susceptible

and resistant populations from rice fields of Arkansas (Arkansas County) were provided by Dr. Jason K. Norsworthy, University of Arkansas.

Seeds from all locations were treated in warm water at 45°C for 20 minutes to break dormancy, then planted and maintained in the greenhouse until plants reached the 3–4 leaf stage to ready for treatment. Day length was 16 h, and the temperature was maintained at 25–28°C during the plant culture period to optimize seedling growth.

DNA extraction

Genomic DNA was isolated from leaf tissue using the DNAzol Reagent protocol (CAS No. 593-84-0, Invitrogen, Thermo Scientific Corp., Waltham, MA). Fresh leaf tissue (1 g) from one represented plant in each population was collected and powdered in liquid nitrogen to prepare for DNA extraction. Each homogenized sample (100 mg) was transferred to a microcentrifuge tube containing DNAzol (1.5 mL) and RNase (150 µL). The solution was mixed and incubated by shaking for 5 min at 25°C. Chloroform (900 µL) was added to the solution and mixed thoroughly for 5 min at 25°C.

The homogenates were centrifuged at 12,000 × g for 10 min, and the aqueous phases were transferred to new tubes. The aqueous phase was mixed with 100% ethanol (700 µL) to precipitate the DNA. The tubes were inverted 6–8 times, held at room temperature for 5 min, and then centrifuged at 5000 × g for 4 min to pellet the DNA. The DNA pellets were washed first in a 1:0.75 (v/v) solution of DNAzol and 100% ethanol (900 µL) by gentle vortexing for 10 s. The DNAzol wash solution was then removed and 75% ethanol (700 µL, prepared with nuclease-free water) was added to the DNA pellets for a second wash step. The mixture was centrifuged at 5000 × g for 4 min. The ethanol wash was decanted and the pellets air dried for 1–2 min. Each DNA sample was solubilized in 50 µL TE buffer, pH 8.0. DNA concentration and purity were determined by spectrophotometric measurement via a NanoDrop instrument (Thermo Scientific Corp, USA).

Random Amplified Polymorphic DNA (RAPD) amplifications

Forty 10-base pair (bp) oligonucleotide primers (synthesized by Integrated DNA Technology, Inc., Coralville, IA) were first screened on the genomic DNA of the 15 *Echinochloa* populations and evaluated for repeatability, high resolution and polymorphism. The PCR master mix contained 2.7 µL ddH₂O, 1 µL PCR buffer (Invitrogen Cat. No. 18067-017),

80 μL MgCl_2 , 5 μM primer, 0.25 μL template DNA, 0.05 μL Taq polymerase (0.5 units), and 1 μL dNTP. The amplification protocol was carried out in a LightCycler 480 II (Roche Molecular Diagnostics, Indianapolis, IN) using the following: initial hold at 94°C for 2 min, followed by 45 cycles of 94°C for 45 s (ramp rate 4.8°C s⁻¹), 38°C for 5 min (ramp rate of 2.5°C s⁻¹), 72°C for 2 min (ramp rate 4.7°C s⁻¹), and a final hold of 72°C for 7 min. Final PCR products were analyzed by LabChip GXII (PerkinElmer, Waltham, MA).

Genetic distance and cluster analyses were conducted for 15 *E. crus-galli* populations using the PCR products of 6 informative primers. For each sample, bands were scored as present (1) or absent (0). The similarity matrix was calculated based on Simple Matching Coefficient (SMC) (Sneath & Sokal 1973). Genetic distance was calculated by the following formula: 1-SMC. We used the NT-SYS 2.1 program to run a cluster analysis to construct an unweighted pair group method average (UPGMA) dendrogram.

Evaluation of herbicide resistance

Plants were grown under the same conditions as used for the DNA extraction and RAPD analysis. At the 3–4 leaf stage, the seedlings were treated with a foliar application of quinclorac (25% SC formulation) at doses that were equal to 31.25, 62.5, 125, 250, 500, 1000 and 2000 g active ingredient (a.i.) ha⁻¹ to calculate the LD₉₀. The experimental design was a completely randomized block, with 4 replications, one pot per replication and 10 plants per pot. Herbicide application was made in a spray booth (Research track sprayer SB-8, Devries Manufacturing, Hollandale, MN); pressure was calibrated to deliver 300 L ha⁻¹ at 140 kPa. At 14 days after treatment (DAT), the mortality rate was assessed by counting the number of surviving

and completely killed plants. The LD₉₀ for each population was calculated by non-linear regression model using GraphPad Prism 7.02 (San Diego, CA) software. Mortality and herbicide rate were fitted into a four parameter logistic curve: $y = a + (a-b)/(1+10^{(\log_{LD90}-x)*e})$ where y: mortality rate; x: quinclorac rate; LD₉₀: estimated dose causing 90% response; a: lower limit; b: upper limit; e: slope around LD₉₀. The R/S ratio at LD₉₀ was calculated by dividing the LD₉₀ value of resistant populations to average LD₉₀ value of susceptible group.

Results and Discussion

RAPD analysis

Among the 40 oligonucleotide primers screened, six primers which produced polymorphic bands and showed repeatable results were selected for the analysis. They were OP-E01, OP-H02, OP-N07, OPH02, DAS04 and DAS08 (Table 1). These primers produced 55 bands, ranging from 50 to 1367 bp; average number of polymorphic bands was 7.7 bands per primer.

Cluster analysis separated the 15 populations into 2 main clusters with a distance between clusters of 0.39 (Fig. 1). Cluster 1 contained 12 populations while Cluster 2 contained only 3 populations. In Cluster 1, there were 5 subclusters. Cluster 1.2 was the largest and contained 6 populations: TG-03, HG-06, HG-02, TG-08, TG-03, CT-04. These populations were closely related, and the within-subcluster genetic distances were 0.17 or less.

Genetic distance between TG-03 and HG-06 populations was the smallest (0.09) among the 15 populations. At a 0.09 genetic distance level, TG-03 and HG-06 may have

Table 1 Six informative primers in RAPD analysis of *Echinochloa crus-galli* populations*

No	Name	Sequence 5'-3'	Number of amplified bands	Number of polymorphic band	Percent of polymorphic band
1	OP-E01	CCCAAGGTCC	8	7	87.5%
2	OP-H02	TCGGACGTGA	10	9	90.0%
3	OP-N07	CAGCCAGAG	9	6	66.7%
4	OP-K20	GTGTCCGAG	9	8	88.9%
5	DAS04	TGAGGAGGAG	10	10	100.0%
6	DAS08	AACGTCTGCC	9	6	66.7%
Total			55	46	-
Average			9.1	7.7	%

*Primers 1-3 were cited from Rutledge et al. 2000, primer 4 was cited in Kim et al. 1998, primers 5-6 were randomly generated.

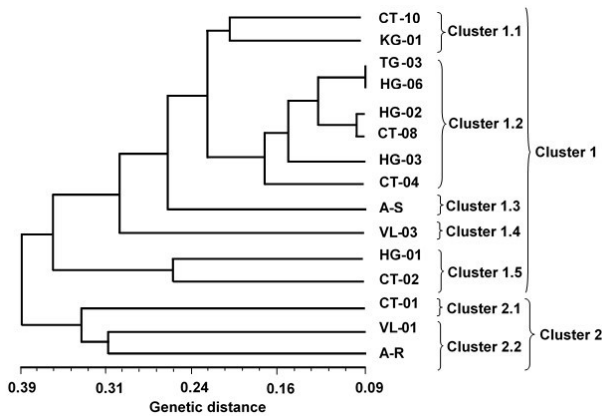


Fig. 1 The dendrogram of 15 *Echinochloa crus-galli* populations from Vietnam (CT-10, KG-01, TG-03, HG-06, HG-02, CT-08, HG-03, CT-04, VL-03, HG-01, CT-02, CT-01, VL-01) and U.S (A-S, AR), the diagram constructed by unweighted pair group method average (UPGMA) and genetic distance calculated by 1-SMC formula

originated from a single population. Geographic distance between the two sampled locations was approximately 130 km (Fig. 2), therefore, they were likely introduced into fields by artificial factors or contamination in rice seeds, however, the origin source is unknown. CT-01 population belonged to Cluster 2 while the other populations in same province (CT-02, CT-04, CT-08, CT-10) were in Cluster 1. In this case, the geographic isolation seemed to have little impact on genetic variation. It is hypothesized that the two populations, Cluster 1 and Cluster 2, were different *Echinochloa* species due to high genetic distance between 2 clusters (0.39) (Hilu 1994). The genetic distance between the two populations from Arkansas was among the highest in this analysis. A-R and A-S were linked in clusters with a 0.39 genetic distance level, suggesting that these two populations might belong to two different species.

Quinclorac resistance

In order to categorize the herbicide resistance levels of these populations, the rating system for S (susceptible) and R (resistant) classification described by Moss et al. (2007) was adopted. The ratings of S, R?, RR and RRR were based on comparing the percentage of weed control at 250 g a.i. ha⁻¹ (recommended labeled dose of quinclorac for barnyardgrass control). Prior to this study, there was no official report estimating the LD₉₀ of *E. crus-galli* in Vietnam therefore, we suggested an average LD₉₀ of 284 g a.i. ha⁻¹ to be the LD₉₀ of the S population.

Locations and results of quinclorac resistance characterization are shown in Fig. 2. There were three populations



Fig. 2 Geographic distribution of 13 *Echinochloa-crus galli* populations collected in the Mekong Delta, Vietnam, one tag represented for one sampled location, different resistant level to quinclorac indicated in different color (green: susceptible; yellow: the resistance maybe involving; red and brown: resistance confirmed)

in category R? (resistance maybe evolving) with R/S ratios of 1.9 ~ 2.2 and percent control at the labeled dose ranging from 73 ~ 76%. The R/S ratios of the four populations in category RR ranged from 2.3 ~ 3.8 with control efficacy of 47 ~ 50% at labeled dose. The highest resistance group RRR contained 4 populations with R/S ratios from 4.9 ~ 6.3, and the labeled dose provided 5-32% weed control within this group.

The most resistant population was CT-02 with a considerably high LD₉₀ value, 1813 g a.i ha⁻¹, indicating that quinclorac at the commercial dose will no longer control this population, higher dose of quinclorac was required to control the RR and RRR populations, therefore, quinclorac use was not economically favored and likely impractical for field weed control of these populations.

Echinochloa diversity

The main target weed of this study at the beginning was *Echinochloa crus-galli*, however the RAPD analysis showed the collected samples based on plant phenotyping were not purely *Echinochloa crus-galli*. From the RAPD analysis of Hilu et al (1994) the genetic distances between different *Echinochloa* species ranged from 0.18 to 0.66. Caton et al. (2003) reported there are three species of *Echinochloa* in Vietnam including *E. colona*, *E. crus-galli* and *E. glabrescens*; on the other hand, Koo et al. (1998) reported only two *Echinochloa* species in the country, *E. colona* and *E. crus-galli*, it is difficult to distinguish *E. crus-galli* from

other *Echinochloa* species because *E. crus-galli* has high phenotypic plasticity, and its morphology tends to be misclassified with other species in the genus, the morphological variation (plant size, reproductive maturity) of *E. crus-galli* is also affected by environment allowing it to adapt to the agricultural practices of the particular geographic region (Tasrif et al. 2004). In addition, two populations of Arkansas *Echinochloa* (A-R and A-S) were separated into 2 main clusters, based on the high genetic distance between these two populations, we speculated that they might belong to two different species within the *Echinochloa* genus. This result agreed with the study of Rutledge et al. (2000) who measured high diversity of *Echinochloa* species in Arkansas rice growing regions in the U.S. where the *Echinochloa* species were separated by RAPD analysis into two distinct clusters with a genetic distance of 0.43. The two populations from Arkansas (A-S and A-R) in this study were different at a 0.39 genetic distance level, however, the A-S population was linked with eight populations in Vietnam at a 0.25 genetic distance level. The results implied those weed populations were related more closely genetically than populations closer in geographical distance. A study by Bonato et al. (2006) in soybean also indicated different soybean cultivars developed for growth in the same geographic area also could not be grouped by genetic similarity (AFLP analysis).

RAPD results and herbicide resistance level

The genetic distance between populations within subclusters and quinclorac resistance level were analyzed in order to find a possible correlation between genetic similarity (RAPD results) and resistance level. In general, genetic distance did not correlate to quinclorac resistance. Within Cluster 1.1, there were two populations of CT-10 and KG-01 linked at 0.22 genetic distance level, but their LD₉₀s differed over 6-fold (1408 versus 210 g a.i ha⁻¹). Similar to Cluster 1, different levels of quinclorac resistance were found in six populations in Cluster 1.2 (genetic distance 0.17). Two groups of TG-03 and HG-06 and HG-02 and CT-08 showed minor differences in genetic distance (0.09 to 0.1, respectively) but the resistance levels to quinclorac were categorized differently between those populations (Table 2). On the other hand, populations that showed similar LD₉₀ exhibited high genetic distance. For example, CT-10 (Cluster 1.1) and A-R (Cluster 2.2) showed similarity in LD₉₀ (1406 and 1487 g a.i ha⁻¹) but were genetically dissimilar; these two populations were linked at 0.39 genetic distance level. Similarly, VL-01 and KG-01, were both identified as same susceptible to quinclorac, but the two populations were distantly related at a 0.39 genetic distance level.

Table 2 Lethal dose of quinclorac needed to kill 90% of the population (LD₉₀) and the Resistant level of 15 *E. crus-galli* populations collected in Vietnam and U.S

Population	Quinclorac LD ₉₀ (g a.i ha ⁻¹)	R/S	% control at 250 g a.i ha ⁻¹	Category*
KG-01	210f	-	97a	S (susceptible) used average LD ₉₀ value of this group (284 g a.i ha ⁻¹) for R/S calculation
VL-01	228f	-	92a	
TG-03	272f	-	88ab	
HG-03	348ef	-	88ab	
CT-08	545e	1.9	76b	R? (resistance may be evolving; possibly reduced herbicide control)
HG-06	558e	1.9	73b	
CT-01	643d	2.2	74b	
CT-04	659d	2.3	50c	RR (resistance confirmed; probably reduced herbicide control)
HG-02	678d	2.4	48c	
A-S	686d	2.4	45c	
HG-01	1087c	3.8	47c	
CT-10	1406b	4.9	32d	RRR (resistance confirmed; highly reduced herbicide control)
A-R	1487b	5.2	18e	
VL-03	1606a	5.6	8e	
CT-02	1813a	6.3	5e	

Means followed by the same letter are not significantly different at $P < 0.05$ (Tukey-Kramer multiple comparison procedure).

*Resistance level rating based on % control at label dose and R rating scale suggested by Moss et al. (2007) where control efficacy at label dose of susceptible (S) is 81 ~ 100%; R? is 72 ~ 80%; RR is 36 ~ 71% and RRR is 0 ~ 35%.

The *Echinochloa* species in the Mekong Delta of Vietnam are diverse. This study showed that populations of *Echinochloa* collected from the same province in Vietnam differed in their susceptibility to quinclorac as well as in genetic similarity. Moreover, the populations exhibiting high quinclorac resistance, regardless of geographic origin, could not be linked by genetic distance analyzed by RAPD. Many species in this genus are misclassified as *Echinochloa crus-galli*, however the quest to name those species remains uncompleted due to the lack of detailed molecular information. Different *Echinochloa* species with different quinclorac resistance levels are growing in a large area, and there could be the coexistence of the resistant and susceptible populations within the same area. The genetic distance results from the RAPD analysis did not provide the needed information to predict quinclorac resistance in *Echinochloa* species, therefore, RAPD analysis was not useful for studying quinclorac resistance in *Echinochloa* spp.; RAPD analysis in this study could be suitable for preliminary evaluating genetic diversity of this weed in Vietnam, other molecular marker and methods should be used to validate the quinclorac resistance and identify species in the required area.

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