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Genetic Variability of *Echinochloa crus-galli* Ecotypes from West Java, Indonesia, by RAPD Analysis

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Abstract

Echinochloa crus-galli (Barnyardgrass: Poaceae), is one of the most detrimental weeds in rice fields globally. As one of the leading rice producers in Indonesia, controlling barnyard grass in rice fields in West Java province is of great importance. Information on the genetic variability of barnyard grass is necessary to determine proper weed control. A molecular marker is considered the most accurate tool in determining genetic variability as its profile is unaffected by the environment. The objective of this study was to evaluate the genetic variability of barnyard grass collected from seven sub-districts in West Java province, Indonesia, using RAPD markers. Genomic DNA of barnyard grass ecotypes from "Bayusari", "Majalaya", "Klari", "Cugenang", "Cianjur", "Ciomas", and "Ciampea" sub-districts were analyzed using eight RAPD primers and resulted in a total of 87 reproducible amplicons. Of these amplicons, 59 were polymorphic, and 28 were monomorphic, with a polymorphism percentage ranging from 37.5-92.8%. Polymorphism information content (PIC) values ranged from 0.21 to 0.41, indicating the used RAPD markers are highly informative. All seven ecotypes were divided into three distinct groups with a coefficient level of 0.77 in a dendrogram constructed following the UPGMA clustering method. Group 1 consisted only of the "Bayusari" ecotype. Group 2 consisted of "Majalaya", "Klari", and "Cugenang" ecotypes, while Group 3 consisted of "Cianjur", "Ciomas", and "Ciampea" ecotypes. This research indicated remote dispersal of *E. crus-galli*, since ecotypes from distant locations were found to be closely related.

Keywords: barnyard grass, invasive weeds, molecular markers, noxious weeds, polymorphism

Introduction

Weed control is important in ensuring sustainable and profitable agricultural production. Competition between weeds and crops in assessing essential growth resources such as water, nutrients, light, and space could significantly reduce crop yields. Additionally, weeds can harbor pests and diseases, further reducing crop health and productivity (Kraehmer et al., 2016). Environmental concerns due to weeds are also rising since weeds can cause significant ecological disruption, such as dominance over native plants and biodiversity reduction (Ramesh et al., 2017). One particularly problematic weed is from the Echinochloa genus (Poaceae) which was found to be frequently associated with rice cultivation (Kraehmer et al., 2016). Barnyard grass (*Echinochloa crus-galli*) is one of the most prevalent Echinochloa species in rice fields. It is considered noxious due to its similar morphology with rice at the seedling stage, high adaptability to broad environmental conditions, and abundance of seed production (Zhang et al., 2021; Sultana et al., 2022; Turra et al., 2023; Vijayakumar et al., 2023). It is an allohexaploid (2n = 6x = 54) annual weedy species with C4 photosynthetic metabolism (Ye et al., 2020; Guo et al., 2017; Zhang et al., 2021; Necajeva et al., 2022). Infestations of *E. crus-galli* in rice fields can reduce yields by 40-50%, depending on weed density and emergence time (Awan et al., 2021). In some extreme cases, yield losses can reach up to 95% (Tian et al., 2020). Furthermore, *E. crus-galli* infestations could lead to higher production costs. The weed's mimicry of rice in its early stages complicates control measures, often leading to delays in management interventions. Studies in Asia calculated increased weed management costs in rice fields infested with *E. crus-galli* (Beltran et al., 2012). These higher production costs and yield losses pose significant economic burdens on farmers.

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One of the most concerning trends related to weed management is the rise of herbicide-resistant weed populations. Continuous use of herbicides, particularly those with the same mode of action, has driven the evolution of resistance in many weed species, including *E. crus-galli* (Damalas and Koutroubas, 2023). Resistant-biotypes of *E. crus-galli* have been documented for various herbicides, including imidazolinone, quinclorac (Matzenbacher et al., 2015), azimsulfuron, and penoxsulam herbicides (Fang et al., 2019; Song et al., 2017). The incidence of herbicide-resistant weeds poses even broader environmental problems globally (Ofosu et al., 2023). In this context, molecular markers have become crucial tools for understanding the genetic basis of herbicide resistance and the development of more targeted and effective management strategies for *E. crus-galli* (Rutledge et al., 2017). In addition to herbicide resistance, molecular markers can also be used to study the genetic diversity and population dynamics of *E. crus-galli* (Božić et al., 2019; Cusaro et al., 2021). Among various molecular markers available to date, Random Amplified Polymorphic (RAPD) DNA markers have become widely used in assessing plant genetic diversity due to their simplicity, cost-effectiveness, and ability to generate rapid results. These markers operate by amplifying random segments of genomic DNA, allowing researchers to detect polymorphisms without prior knowledge of the genome sequence (Amiteye, 2021). In this study, we evaluated the genetic variability of *E. crus-galli* collected from seven sub-districts in West Java province, Indonesia, using RAPD markers. The information on genetic diversity among different *E. crus-galli* ecotypes identified in this study should help researchers track the spread of specific ecotypes, evaluate the effectiveness of management practices, and develop region-specific weed management plans.

Material and Methods

Genetic Materials and Sample Collection

Samples of *E. crus-galli* were collected in July 2023 from two to three sub-districts from three districts in West Java province, namely Karawang, "Cianjur," and Bogor, resulting in a total of seven locations, as shown in Figure 1. Approximately 20 *E. crus-galli* flowers containing physiologically mature seeds were collected from at least five points in each location, with a minimum distance of 1 m and a maximum distance of 10 m between sampling points. Flowers were sun-dried for two days, and seeds were collected and stored at room temperature. Seeds were planted in January 2024. Seeds were immersed in 2.5 ppm

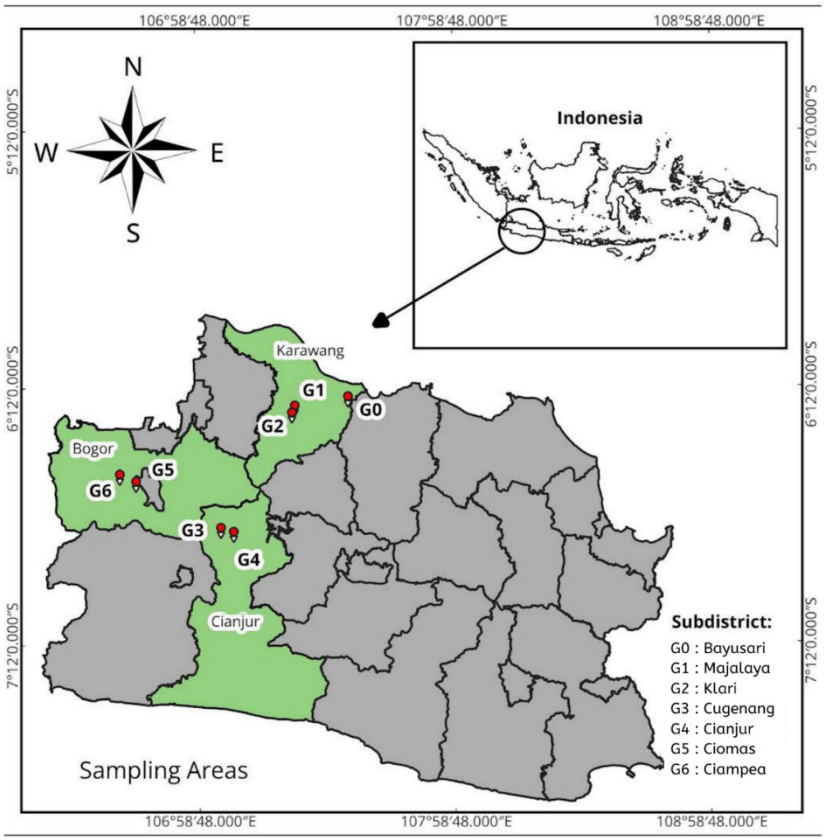

Figure 1. Sampling sites of *E. crus-galli* ecotypes in West Java, Indonesia.

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gibberellic acid (GA_3) for 10 minutes and planted on 18 cm diameter plastic pot containing soil containing 30 seeds per pot. Plants were maintained in a 40% shade house at Cikabayan Bawah Experimental Field, IPB University (±186 m asl) for further application.

DNA Isolation and PCR Condition

Leaves of a one-month-old plant were harvested and preserved in a 2-mL microtube containing 700 µL CTAB (Cetyl-trimethyl ammonium bromide) buffer and stored at -20°C. Genomic DNA was isolated from the preserved leaves following the CTAB method (Aboul-Maaty dan Oraby, 2019) with slight modification. DNA integration was evaluated by agarose gel electrophoresis (1.5%, w/v; 1x TAE, 90 volts, 45 minutes). DNA concentration and potential contamination were evaluated using the Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, USA). Genomic DNA was diluted into 12 ng.µL^{-1} concentration for further application as a template in PCR.

Eight 10-mer oligonucleotides with random sequences were used in RAPD analysis (Table 1). The DNA fragments amplification was performed in a final volume of 10 µL, consisting of 2.5 µL of genomic DNA (12 ng.µL^{-1}), 2.5 µL of primer (10 pmol), and 5.0 µL of 2× PCR mix (MyTaq HS Red Mix). PCR was performed using Esco's Swift Maxi Thermal Cycler (Esco Technologies, Singapore). Amplification conditions were 94°C for 5 minutes for pre-denaturation, followed by 45 cycles of 5 seconds at 94°C, 30 seconds at 47°C for annealing, and 1 minute at 72°C for extension. A final extension at 72°C for 10 minutes was set at the end of the cycle. The amplified DNA fragments were analyzed by electrophoresis at 90 volts for 85 minutes in 1x TAE buffer on 1.5% (w/v) agarose gel. The low reproducibility of RAPD markers is a well-recognized limitation. However, several strategies can be employed to improve the reproducibility of RAPD markers, including the utilization of proper replications (Ramos et al., 2008). Therefore, to assess the reproducibility of the profiles, the PCR procedures were replicated four times. Gels were further stained in an ethidium bromide solution (0.5 µg.mL^{-1}) and were visualized using a UV transilluminator (AlphaImager® Mini). The gel image was analyzed using GelAnalyzer 23.1 with a band intensity threshold value of 55.

The RAPD bands were scored as "1" for the presence or "0" for the absence of a particular DNA fragment of a similar size. Only reproducible and clear amplification bands were scored for constructing the data matrix. The data were entered into NTSYS-pc, a numerical taxonomy and multivariate analysis system program (Rohlf, 1998). The 0/1 matrix was used to calculate the similarity in the matrices using 'Simqual', a subprogram of the NTSYS-pc software. The dendrogram was built based on the unweighted pair group method with the Bootstrap value of 1,000.

Result and Discussion

The genetic diversity of seven *E. crus-galli* ecotypes from West Java, Indonesia, was analyzed using eight RAPD markers. Primer selection is crucial in genetic diversity analysis using RAPD markers because it influences the results' accuracy, reproducibility, and informativeness (Amiteye, 2021). In this study, primers A7, A20, E1, and H2 were selected according to the study of Rutledge et al. (2017), who assessed the genetic variation of *E. crus-galli* populations in Arkansas with different resistance to propanil herbicide. Meanwhile, primers E2, H13, M17, and M24 were selected as they were highly informative in the genetic variability assessment of Indonesian foxtail millet (*Setaria italica* L. Beauv) genotypes (Ardie et al., 2017). High synteny between *E. crus-galli* genome and *S. italica* was previously reported by (Ye et al*.,* 2020). These primers successfully resulted in reproducible amplicons (Table 1). The size of the amplified products ranged from 100-2,700 bp and the total number of bands produced ranged from 7 to 14 bands per primer. A total of 87 amplicons were produced, of which 59 were monomorphic, and 28 were polymorphic. The number of polymorphic bands for each primer varied from 3 (primer M24) to 13 (primer A7). Primers E2, H13, A7, A20, E1, H2, A20, and E1 produced more polymorphic bands (7-13) than M17 and M24, which produced 3 and 4 polymorphic bands, respectively. The calculated polymorphism percentage varied from 37.50% (primer M24) to 92.86% (primer A7). The representative RAPD profiles produced by primers A7 and M24 are presented in Figure 2. The polymorphism percentage represents the proportion of polymorphic bands relative to the number of amplified bands across samples. Thus, it indicates genetic diversity among the samples studied. Seven primers in this study showed a polymorphism percentage of more than 50%, indicating a considerably high variation among the eight *E. crus-galli* ecotypes.

Polymorphism information content (PIC) measures the capacity of a marker to identify the polymorphism among tested individuals. The PIC value for dominant markers ranges from 0 to 0.5. The informativeness for dominant markers can be classified based on their PIC values as low (0-0.10), medium (0.10-0.25), high (0.30-0.40), and very high (0.40-0.50) (Serrote et al. 2020). The results of this study indicate that the PIC

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Table 1. List of RAPD primers, band size range, number of amplified bands, the polymorphism percentage, and polymorphism information content (PIC) of seven *E. crus-galli* ecotypes from West Java, Indonesia

Primer	Sequence (5'-3')	Band size (bp) min-max	Polymorphic bands	Monomorphic bands	Total bands	Polymorphism percentage (%)	PIC
E2	GGTGCGGGAA	300-2,000	8	4	12	66.67	0.30
H13	GACGCCACAC	150-1,800	7	6	13	53.85	0.26
A7	GAAACGGGTG	100-1,500	13	1	14	92.86	0.41
A20	GTTGCGATCC	200-1,300	7	4	11	63.64	0.31
E1	CCCAAGGTCC	200-2,700	9	4	13	69.20	0.33
H2	TCGGACGTGA	200-2,000	8	2	10	80.00	0.35
M17	TCAGTCCGGG	300-1,300	4	3	7	57.14	0.21
M24	GGCGGTTGTC	400-1,400	3	5	8	37.50	0.23

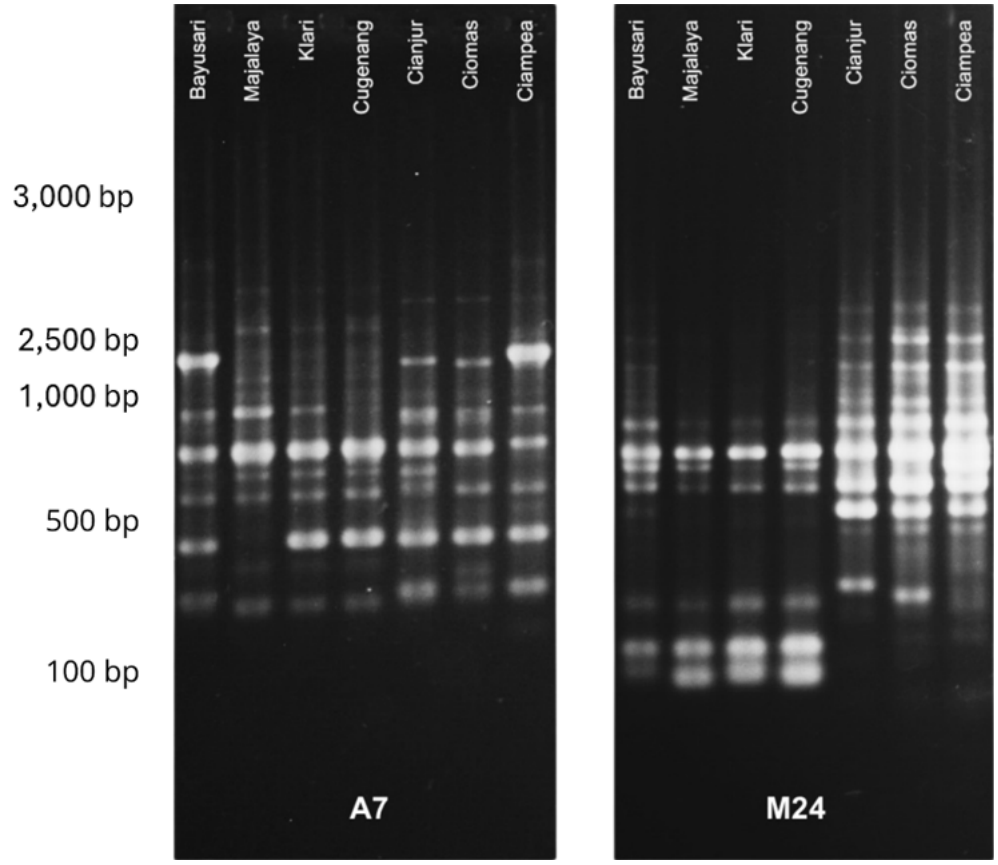

Figure 2. Representative RAPD profiles of seven *E. crus-galli* ecotypes with the highest (A7 primer) and the lowest (M24 primer) polymorphism percentage.

values for the primers tested ranged from 0.21 to 0.41. Primer A7 had the highest PIC value, at 0.41, falling into the very high category, while primer M24 had the lowest, at 0.21, which is classified as medium. Most of the primers exhibited high PIC values, suggesting that these markers are highly informative for detecting genetic variation among the *E. crus-galli* ecotypes.

The polymorphism percentage and the PIC value suggested that RAPD primers used in this study could sufficiently discriminate the seven *E. crus-galli* ecotypes. Therefore, a dendrogram was better constructed to visualize the genetic relationship among the seven ecotypes. The UPGMA clustering method placed all seven ecotypes in three distinct groups at a coefficient level of 0.77 (Figure 3). Group 1 consisted only "Bayusari" ecotype. Group 2 consisted of "Majalaya", "Klari", and "Cugenang"

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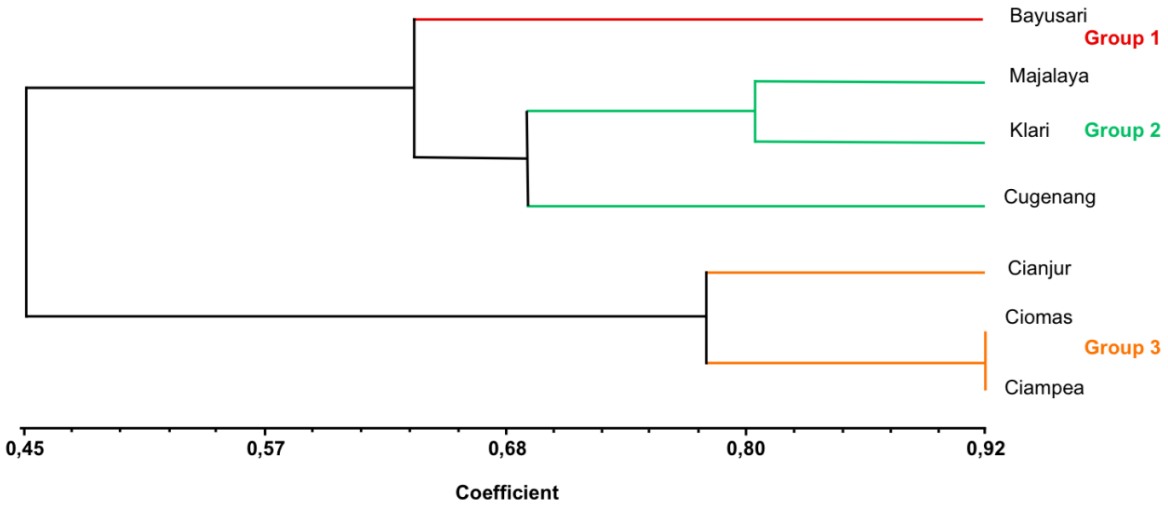

Figure 3. The dendrogram of seven *E. crus-galli* ecotypes from West Java, Indonesia, constructed by unweighted pair group method average (UPGMA).

ecotypes, while Group 3 consisted of "Cianjur", "Ciomas", and "Ciampea" ecotypes. The genetic diversity analysis using AFLP markers on *E. crus-galli* from diverse origins by Danquah et al. (2002) indicated that the genetic variability represents the geographical origin. However, it is important to note that in this study some *E. crus-galli* ecotypes from distant geographical locations were grouped in the same cluster. For example, the "Cianjur" ecotype was found to be closely related to the"Ciampea" and "Ciomas" ecotypes from the Bogor Regency. Meanwhile, the "Cugenang" ecotype from "Cianjur" regency was grouped with the "Majalaya" and "Klari" ecotypes from the Karawang regency. This finding indicates the potential of *E. crus-galli* dispersal to distant locations. Major weed species of rice are commonly dispersed by water, animals, and human activities (Shekhawat et al., 2020). However, distant dispersal seems to be facilitated by contaminated rice seeds. Depending on the emergence time, some *E. crus-galli* ecotypes mature simultaneously with rice (Vijayakumar et al., 2023), thus increasing the possibility of being mixed with rice seeds during harvesting. Our result highlights the importance of rice weed management, with particular emphasis on *E. crus-galli*. Herbicide-resistant E. *crus-galli* incidences have been increasingly reported (Matzenbacher et al., 2015; Song et al., 2017; Fang et al., 2019); thus, the dispersal of these resistant ecotypes would surely harm rice productivity. In this regard, herbicide resistance of the seven *E. crus-galli* ecotypes assessed in this study should be further investigated.

Conclusion

Eight 10-mer oligonucleotides used in this study showed sufficient discriminant capacity for seven *E. crus-galli* ecotypes with polymorphism percentages ranging from 37.50% to 92.86%, and PIC values ranging from 0.21 to 0.41. The UPGMA clustering method placed the seven ecotypes into three distinct groups at the coefficient level of 0.77, namely group 1 ("Bayusari" ecotype), group 2 ("Majalaya", "Klari", and "Cugenang" ecotypes), and group 3 ("Cianjur", "Ciomas", and "Ciampea" ecotypes). Some *E. crus-galli* ecotypes from distant locations were found to be closely related, indicating remote dispersal of the weed.

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