[BIO05] Genetic structure of glyphosate-resistant (R) and glyphosate-susceptible (S) populations of *Eleusine indica* (L.) Gaertn from Peninsular Malaysia

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Introduction

Glyphosate is the world's most widely used herbicide, accounting for 11 % of worldwide herbicide sales (Powles *et al*., 1997). As a nonselective herbicide with no soil activity (Grossbard & Atkinson 1985), it is an ideal herbicide to control a broad range of weed species. In Malaysia, glyphosate is used to control various weed species growing in oil palm and rubber plantations. In some instances, multiple treatments have been carried out continuously for several years. However, this intensive usage has led to the appearance of resistant *Eleusine indica* populations in Malaysia.

Eleusine indica (L.) Gaertn, commonly known as goosegrass is an annual, self-pollinating cosmopolitan diploid grass of the Poaceae (Gramineae) family (Barnes & Chan 1990). Although used as animal feed and a source of grain in some regions, it is considered one of the five "world's worst weeds" and has been reported to be a problem weed in 46 different crop species in more than 60 countries (Holm *et al*., 1977). In Malaysia, it is one of the most serious weeds in vegetable farms, orchards, oil palm and rubber plantations, as well as in wasteland and along roadsides (Holm *et al*., 1977).

Control efforts of the R biotype of *E. indica* have focused on eradication with limited success. Thus, quantifying genetic variation and understanding the genetic structure of this weed will improve our ability to better predict the success of weed management strategies, providing an environmentally sound and more cost effective approach to managing *E. indica*.

Materials and methods

Plant materials

Mature *E. indica* seeds from six areas in Peninsular Malaysia that were reported to be glyphosate-resistant and glyphosate-susceptible namely Bidor, Chaah, Lenggeng, Temerloh, Kuala Selangor and Melaka were collected in addition to two susceptible populations from Sungai Tangkas, Selangor and Pulau Pinang. Inflorescence of 10 individuals consisting of either 10 putative glyphosate-resistant and/ or 10 putative glyphosate-susceptible individuals from each area were collected and placed in different envelopes. The seeds collected were then germinated in polybags in the greenhouse at the Plant Biotechnology Laboratory, UKM for screening of the S and R biotypes. The polybags were placed according to blocks where each block represents the S and R biotype from each area. To avoid cross contamination, the blocks were isolated by a boundary of about 1 meter from each other. Screenings of the R and S biotypes were then done by applying glyphosate at the recommended dosage of 1.08 kg ae/ha on the seeds. Germinated seeds were allowed to reach maturity and inflorescences of each mature individual were collected and kept.

14 populations, 6 of the R biotype and 8 S populations were used for this study and approximately 840 accessions were sampled, consisting of 30 samples from each population. Table 1 shows the summary of populations and number of samples used for isozyme analysis.

Enzyme extraction, Isozyme electrophoresis and staining

Enzymes were extracted from young shoots of two month old plants. Crude extracts for the electrophoresis were prepared from fresh young leaf samples with a ratio of 1:1 of leaf weight to the extraction buffer. The methodology used is according to Wickneswari & Norwati (1991).

After electrophoresis, the gel was sliced horizontally and assayed for 25 enzymes. These enzymes are as follows: aspartate aminotransferase (AAT) [E.C. 2.6.1.1.], esterase

(EST) [E.C. 3.1.1], glucose -6- phosphate isomerase (GPI) [E.C. 5.3.1.9], peroxidase (PER) [E.C. 1.11.1.7], catalase [CAT, E.C.1.11.1.6.], hexokinase [HK, E.C. 2.7.1.1.], leucine-aminopeptidase [LAP, E.C.3.4.11.1.], peptidase [PEP, E.C.3.4.-.-.], aconitase hydratase [ACO, E.C.4.2.1.3.], acid phosphate (ACP) [E.C. 3.1.3.2], fructose-biphosphate aldolase (FBA) [E.C. 4.1.2.13] , isocitrate dehydrogenase (IDH) [E.C. 1.1.1.42], malate dehydrogenase (MDH) [E.C. 1.1.1.37], malic enzyme (ME) [E.C. 1.1.1.40], shikimate dehydrogenase (SKDH) [E.C. 1.1.1.25], 6-phosphogluconate dehydrogenase [6PGD, E.C.1.1.1.49], shikimate dehydrogenase [SKDH, E.C.1.1.1.25], glutamate dehydogenase (GDH) [E.C. 1.4.1.2], glycerate dehyrogenase (GLY) [E.C. 1.1.1.29], phosphoglucomutase (PGM) [E.C. 2.7.5.1], triosephosphate isomerase (TPI) [E.C. 5.3.1.1], uridine diphosphogluconate pyrrophosphatase (UGP), succinate dehydrogenase (SUDH)[E.C.1.3.99.1.], diaphorase (DIA), and menadione reductase (MR). The enzyme staining methods were modified from Wickneswari & Norwati (1991) and Soltis *et al*. (1983).

Data analysis

Due to the lack of consistent activity, only 8 enzyme systems were used for this study namely glutamate dehydrogenase (GDH), glucose-6-phosphate isomerase (GPI), phosphoglucomutase (PGM), acid phosphatase (ACP), isocitrate dehydrogenase (IDH), glycerate dehydrogenase (GLY), uridine diphosphogluconate pyrophosphate (UGP) and malate dehydrogenase (MDH). Full-sib progeny arrays were used to infer the genetic basis of complex banding patterns such as MDH, and ACP.

The isozyme data were analyzed using the computer programme POPGENE version 1.32 (Yeh & Boyle 1999) and BIOSYS-2 (Swofford & Selander 1997). Parameters of genetic diversity estimated using POPGENE included allelic frequencies, mean number of alleles per locus (A_e) which was calculated according to Crow & Kimura (1970), percentage of polymorphic loci (0.99 criterion) (P), expected (H_e) and observed (H_o) proportion of heterozygosities, genetic differentiation (F_{st}) and fixation indices (F_{is}) and their variances. BIOSYS-2 was used to test for deviation from Hardy-Weinberg equilibrium and to calculate genetic distance (Nei 1978). The values were then used to generate a dendrogram using the unweighted pair-group with arithmetic average (UPGMA) cluster analysis as described by Sneath & Sokal (1973).

TABLE 1 Summary of the *E. indica* populations used for isozyme analysis

Population	Geographical Coordinates (in degrees minutes seconds)	Biotype	Population number
Bidor	4° 07’ 00”, 101° 17’ 00”	Susceptible	1
		Resistant	2
Chaah	2° 14’ 00”, 103° 02’ 00”	Susceptible	3
		Resistant	4
Lenggeng	2° 52’ 00”, 101° 56’00”	Susceptible	5
		Resistant	6
Temerloh	3° 27’ 00”, 102° 25’ 00”	Susceptible	7
		Resistant	8
Kuala Selangor	3° 21’ 00”, 101° 15’ 00”	Susceptible	9
		Resistant	10
Melaka	2° 11’ 49”, 102° 14’53”	Susceptible	11
		Resistant	12
Sungai Tangkas	2° 54’ 00”, 101° 47’ 00”	Susceptible	13
Pulau Pinang	3° 33’ 00”, 102° 34’ 00”	Susceptible	14

Results and discussion

Allele frequencies

For this study, 14 populations were tested namely Bidor R and S, Chaah R and S, Lenggeng R and S, Temerloh R and S, Kuala Selangor R and S, Melaka R and S, and Sungai Tangkas S and lastly Pulau Pinang S (Table 1). A total of 8 enzyme systems were assayed, in which 13 loci were detected, which included GDH-1, GLY-1, UGP-1, ACP-1, GPI-1, GPI-2, IDH-1, IDH-2, IDH-3, PGM-1, PGM-2, MDH-1 and MDH-2. From these, a total of 3 loci were found to be polymorphic namely ACP-1, MDH-1 and MDH-2.

This apparent lack of diversity is similar to many weedy, predominantly self-pollinated species such as *Avena fatua*, *Elymus* spp., *Hordeum* spp., and *Lolium* spp (Hamrick et al. 1979). This may be due to the fact that regardless of wide geographic ranges, the disturbed habitats of weeds do not offer much variation site-to-site. Thus a limited number of highly plastic general-purpose variants within a weed species may suffice for adaptation to its spatially homogeneous but temporally heterogeneous environments (Baker 1965). To a certain extent, allelic frequency exhibited in the *E. indica* populations surveyed may be due to reproductive isolation and genetic drift.

TABLE 2 Allele frequencies in 14 populations

Locus	Allele	Population/ biotype													
		Bidor		Chaah		Lenggeng		Temerloh		K.Slgr		Melaka		S.Tangkas	P.P.
		S	R	S	R	S	R	S	R	S	R	S	R	S	S
Gly-1	a	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gdh-1	a	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ugp-1	a	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pgi-1	a	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pgi-2	a	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pgm-1	a	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pgm-2	a	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Idh-1	a	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Idh-2	a	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Idh-3	a	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acp-1	a	0.033	0.033	0.433	0.533	0.400	0.433	-	0.133	0.933	0.167	0.967	0.133	0.967	0.967
	b	0.967	0.967	0.567	0.467	0.600	0.567	1	0.867	0.067	0.833	0.033	0.867	0.033	0.033
Mdh-1	a	-	-	-	-	0.100	0.033	0.330	-	-	-	-	-	-	-
	b	1	1	1	1	0.900	0.967	0.667	1	1	1	1	1	1	1
Mdh-2	a	0.033	0.133	0.050	0.350	0.450	0.317	0.967	0.767	-	0.733	-	0.850	-	-
	b	0.967	0.867	0.950	0.650	0.550	0.683	0.033	0.233	1	0.267	1	0.150	1	1

TABLE 3 Mean of genetic variability parameters per biotype (standard errors in parentheses)

Biotype	A	A_e	P	H_o	H_e	F_{is}	F_{st}	N_m
Susceptible	1.2	1.1	23.1	0.014 (0.051)	0.069 (0.152)	0.876	0.622	0.152
Resistant	1.2	1.1	23.1	0.003 (0.12)	0.067 (0.067)	0.724	0.240	0.790

Genetic variability measures

Table 3 and 4 shows genetic variability measures by biotype and across all populations respectively. In general, very low genetic diversity was observed. Across the populations, the mean number of allele per locus (A) was found to be small (1.2), while the mean effective number of alleles (A_e) was 1.1. The range of percentage of polymorphic loci (P) at 0.95 criterion was from 0.00 to 23.1 % with a mean of 9.9 %.

As per biotype (Table 3), the mean percentage of polymorphic loci (P= 23.1 %), mean number of alleles per locus (A= 1.2) and effective number of alleles per locus (A_e= 1.1) was similar for both the R and S populations. Levels of expected heterozygosity (H_e) was low and not significantly different (P >0.10) between the R (H_e=0.067) and S (H_e= 0.069) biotypes but the levels of observed heterozygosity (H_o) was found to be significantly lower (P<0.10) in the R populations (H_o=0.003) than in the S populations (H_o=0.014). The low genetic variation within populations is consistent with the autogamous reproduction in *E. indica*. The reduced level of genetic variation in the R populations may be due to the effects of genetic drift associated with the selection for herbicide resistant individuals and the subsequent build-up of homogenous resistant populations.

TABLE 4 Genetic variability at 13 loci in all populations (standard errors in parentheses)

Population	Biotype	N	A	A_e	P	H_o	H_e	F_{is}
Bidor	Susceptible	30	1.2	1.0	0	0.005 (0.005)	0.010 (0.007)	0.483
	Resistant	30	1.2	1.0	7.7	0.010 (0.010)	0.023 (0.018)	0.549
Chaah	Susceptible	30	1.2	1.1	15.4	0.008 (0.008)	0.046 (0.039)	0.821
	Resistant	30	1.2	1.1	15.4	0.028 (0.028)	0.075 (0.051)	0.615
Lenggeng	Susceptible	30	1.2	1.2	23.1	0.008 (0.008)	0.090 (0.052)	0.913
	Resistant	30	1.2	1.1	15.4	0.013 (0.013)	0.077 (0.049)	0.831
Temerloh	Susceptible	30	1.2	1.1	7.7	0.005 (0.005)	0.040 (0.035)	0.869
	Resistant	30	1.2	1.1	15.4	0.005 (0.005)	0.046 (0.032)	0.887
Kuala Selangor	Susceptible	30	1.1	1.0	7.7	0 (0.00)	0.010 (0.010)	1.0
	Resistant	30	1.2	1.1	15.4	0.010 (0.010)	0.052 (0.036)	0.801
Melaka	Susceptible	30	1.1	1.0	0	0 (0.00)	0.005 (0.005)	1.0
	Resistant	30	1.2	1.0	15.4	0.018 (0.018)	0.038 (0.026)	0.520
Sungai Tangkas	Susceptible	30	1.1	1.0	0	0 (0.00)	0.005 (0.005)	1.0
Pulau Pinang	Susceptible	30	1.1	1.0	0	0 (0.00)	0.005 (0.005)	1.0
MEAN:			**1.2**	**1.1**	**9.9**	**0.001**	**0.037**	**0.806**

Fixation indices

In this study, F_{is} values were found to range from 0.4956 to 1.0 with a mean of 0.785. The lowest F_{is} value was from MDH-2 while the other two loci, ACP-1 and MDH-1 had a F_{is} value of 1.00 respectively (Table 5). All the populations surveyed had a positive value of F_{is} (Table 4), indicating an excess of homozygotes, which may have been caused by small populations sizes, or inbreeding. This agrees with Wahlund (1928) whom stated that genotype frequencies in natural populations that are divided into sub-populations might deviate from Hardy-Weinberg equilibrium due to natural selection on in the case of small populations, random genetic drift.

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TABLE 5 Summary of F-statistics and gene flow at polymorphic loci

Loci	Sample size	F_{is}	F_{it}	F_{st}	N_m*
ACP-1	840	1.0	1.0	0.5435	0.2100
MDH-1	840	1.0	1.0	0.2365	0.8073
MDH-2	840	0.4956	0.7692	0.5425	0.2108
MEAN	840	0.785	0.898	0.523	0.228

Genetic differentiation among populations

Among the *Eleusine indica* populations surveyed, overall degree of genetic differentiation was 0.523(Table 5). This indicates that there was high divergence among the populations. The partitioning of genetic diversity per biotype showed that the F_{st} values among the R populations were low (0.240) while the S populations had a high F_{st} value (0.622), which contributed to the high genetic differentiation among the 14 populations (Table 3).

Between loci, the F_{st} values were significantly different, ranging from 0.2365 for MDH-1 to 0.5435 for ACP-1, as shown in Table 5.

Principally, genetic differentiation among populations is a function of gene flow among populations by pollen and seed dispersal. Populations that are isolated will experience less gene flow than contiguous or continuously distributed populations. Low levels of gene flow between populations will therefore be consistent with higher levels of genetic differentiation among populations.

For the populations surveyed, it was found that the total gene flow across the populations was low at N_m of 0.2247. The consequences of gene flow and genetic bottlenecks on the genetic structures of certain populations include possible results such as decreased fitness through outbreeding or inbreeding depression and reduction of local variation. The eventual outcome of this process will be homogenization of allele frequencies and a severe reduction in genetic diversity among populations.

TABLE 6 Estimates of mean genetic distance (Nei 1978) between 14 populations of *E. indica*

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 Bidor S	*													
2 Bidor R	0.000	*												
3 Chaah S	0.028	0.029	*											
4 Chaah R	0.027	0.023	0.007	*										
5 Lenggeng S	0.024	0.019	0.017	0.002	*									
6 Lenggeng R	0.018	0.015	0.008	0.000	0.000	*								
7 Temerloh S	0.080	0.066	0.114	0.064	0.039	0.058	*							
8 Temerloh R	0.044	0.033	0.063	0.026	0.014	0.023	0.013	*						
9 K.Slngor S	0.065	0.067	0.007	0.022	0.039	0.027	0.163	0.102	*					
10 K.Slngor R	0.040	0.030	0.056	0.022	0.011	0.019	0.015	0.000	0.093	*				
11 Melaka S	0.070	0.072	0.008	0.024	0.042	0.030	0.169	0.106	0.000	0.097	*			
12 Melaka R	0.054	0.042	0.073	0.033	0.018	0.030	0.011	0.000	0.113	0.000	0.118	*		
13 S.Tgkas S	0.070	0.072	0.008	0.024	0.042	0.030	0.169	0.106	0.000	0.097	0.000	0.118	*	
14 P.Pinang S	0.070	0.072	0.008	0.024	0.042	0.030	0.169	0.106	0.000	0.097	0.000	0.118	0.000	*

Genetics relatedness

Genetic distance among populations was very low and the mean genetic distance for the 14 *E. indica* populations surveyed was 0.046, with values ranging from 0.000 to 0.169 (Table 6). The lowest genetic distance value is at 0.00. This value was observed was between several pairs of populations namely between Bidor S and Bidor R, Chaah R and Lenggeng R, Lenggeng S and Lenggeng R, Temerloh R and Kuala Selangor R, Temerloh R and Melaka R, Kuala Selangor S and Melaka S, Kuala Selangor S and Sungai Tangkas S, Kuala Selangor S and Pulau Pinang S, Kuala Selangor R and Melaka R and Sungai Tangkas S and Pulau Pinang S.

The highest value of genetic distance was instead observed between Temerloh S and Melaka S, Temerloh S and Sungai Tangkas S, and also between Temerloh S and Pulau Pinang S. It is apparent that genetic distance between many pairs of *E. indica* populations did not correlate with the geography of the populations. It is possible that human activities transported seeds from one region to another, in addition to other genetic diversity influencing factors such as founder events, genetic drift and others.

The UPGMA dendrogram in Figure 1 shows how the populations are grouped on the basis of Nei's (1978) genetic distance. There are two main clusters; cluster I consisting of all the R populations and S populations from Bidor, Chaah, Lenggeng and Temerloh while cluster II has only S populations from Kuala Selangor, Melaka, Sungai Tangkas and Pulau Pinang.

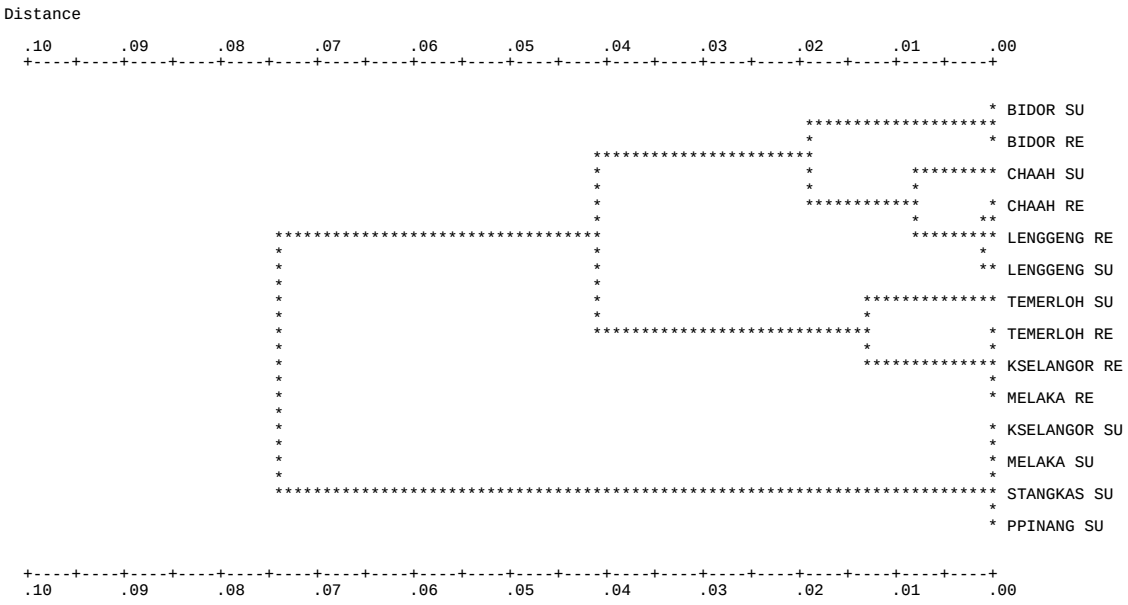

FIGURE 1 A dendrogram based on UPGMA clustering of *Eleusine indica* populations using Nei's (1978) genetic distance.

Conclusion

The reproductive strategy of a plant species is a key factor affecting both the level and organization of genetic variation within and among populations. In general, predominantly autogamous species are characterized by substantially greater genetic differences among populations and reduced genetic variability within populations (Hamrick & Godt 1990). This was clearly shown in the high value of genetic differentiation among populations of *E. indica* which is a selfing species.

The genetic structure of *E. indica* populations would appear to differ with biotype. Thus, the number of years since resistance has appeared in a weed population and whether or not glyphosate has been used regularly after the resistance appeared will also affect the levels of genetic polymorphism in resistant populations. Evidence suggests that *E. indica* populations from founder glyphosate-resistant plants remain genetically less variable as compared to the wild type (susceptible) populations.

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