JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Morphological and Molecular Characterization of Different *Echinochloa* spp. and *Oryza sativa* Populations

J. P. RUIZ-SANTAELLA,*,†,§ F. BASTIDA,# A. R. FRANCO,§ AND R. DE PRADO†

Department of Agricultural Chemistry and Edaphology and Departament of Biochemistry and Molecular Biology, University of Córdoba, Córdoba, Spain; and Department of Forestry and Agricultural Sciences, University of Huelva, Huelva, Spain

Echinochloa P. Beauv. is an important genus because many of its species are weeds infesting most paddy fields, which can reduce the rice grain production by up to 80%. A controversy exists about the taxonomy of the genus due to the high level of morphological variations found in these species. Cyhalofop-butyl, an aryloxyphenoxy-propionate herbicide, is used to control *Echinochloa* spp. in paddy fields, although differences in susceptibility were found between different *Echinochloa* species. *E. colona* was highly susceptible [ED_{50} = 34 g of active ingredient (ai) ha^{-1}]; very similar results were obtained with the remaining species. By contrast, *E. oryzicola* (170 g of ai ha^{-1}) was less sensitive, with the herbicide symptoms appearing later. Because of this differential susceptibility, morphological and molecular studies were carried out. A morphological study, using 21 characters both quantitative and qualitative of spikelets and seedlings, was capable of clearly distinguishing closely related *E. crus-galli* plants (two populations), *E. muricata* and *E. crus-pavonis*, and *E. oryzicola*, *E. utilis*, and *E. colona* species. The resolution of *Echinochloa* species at the molecular level, based on RAPD analyses, was fairly consistent with morphological analysis results. Among the 60 primers screened, 21 primers exhibited polymorphic bands and produced a total of 136 RAPD markers. Of all the amplified fragments, 90 were found to be polymorphic. *E. oryzicola* and *E. colona* were clearly separated, and the RAPD analyses showed that both *E. crus-galli* populations were 100% related and 51% related to *E. utilis*, whereas *E. crus-pavonis* and *E. muricata* (73% similarity) appeared as being clearly separated from this group.

KEYWORDS: ***Echinochloa*; *Oryza sativa*; RAPD analyses; cyhalofop-butyl (CB)**

INTRODUCTION

The genus *Echinochloa* P. Beauv. belongs to the tribe Paniceae R. Br. subfamily Panicoideae A. Br., family Gramineae Juss (= Poaceae Barnh.). There is some disagreement about the species that constitute *Echinochloa*. The genus may include 20−50 annual and perennial wild species that are widely distributed in tropical and warm temperate regions of the world (*1*−*4*). Many of them are among the world's most important warm-season annual grass weeds (*5*, *6*), especially in paddy fields or swampy places, causing serious competition with the crop and reducing its yield (*7*, *8*). A correct species identification is agronomically and economically important because *Echinochloa* spp. are aggressive invaders that are difficult to control. Nevertheless, many species are hard to distinguish because they tend to intergrade (*9*−*11*). Some of the characters traditionally used for distinguishing taxa, for example, awn length, show a high degree of phenotypic plasticity in response to environmental conditions; others reflect selection by cultivation, for example, nonshattering or mimicry of rice in paddy fields. Farmers have to use herbicides to control these weeds by means of pre- and postemergence treatments. Due to the specific conditions required for the development of this crop, with a permanent layer of water in many cases, the number of herbicides available to control weeds in paddy fields is not as large as in other crops. Cyhalofop-butyl (CB), 2-[4-(4-cyano-2-fluorophenoxy)phenoxy]propionic acid, butyl ester (*R*), is an aryloxyphenoxy-propionate herbicide for the postemergence control of grasses in paddy fields at application rates of 300 g of active ingredient (ai) ha^{-1}, mainly against almost all *Echinochloa* species. Like other aryloxyphenoxy-propanoate (AOPP) and cyclohexanedione (CHD) herbicides, the site of action of CB is acetyl coenzyme A carboxylase (ACCase, EC 6.4.1.2), an enzyme catalyzing the first committed step in de novo fatty acid biosynthesis (*12*). Different herbicide susceptibilities between species showed the need to establish suitable methods for discriminating genotypes and resolving taxonomic relationships within *Echinochloa* (*13*, *14*). In this regard, DNA-based molecular markers are particularly useful for identifying

* Corresponding author (telephone +34 957 218 600; fax +34 957 218 830; e-mail qe2rumoj@uco.es).
† Department of Agricultural Chemistry and Edaphology, University of Córdoba.
§ Department of Biochemistry and Molecular Biology, University of Córdoba.
# University of Huelva.

10.1021/jf0520746 CCC: $33.50
Published on Web 01/27/2006

Table 1. Description, Origins, Countries, and Crop/Areas of *Echinochloa* and *O. sativa* Populations

description	origin, country	crop/area
O. sativa Bahia cv.	Seville, Spain	rice
O. sativa (weedy rice)	Badajoz, Spain	rice
E. crus-galli 6	Stuttgart, Germany	maize
E. crus-galli 7	Stuttgart, Germany	maize
E. muricata	Sacramento, CA	wetlands
E. oryzicola	Seville, Spain	rice
E. utilis	Yamagata, Japan	rice
E. crus-pavonis	Seville, Spain	rice
E. colona	Tanga, Tanzania	vegetable fields

genetic diversity within plant species and characterizing closely related genotypes (*15*). RAPD markers are produced using the Polymerase Chain Reaction (PCR) when short primers of arbitrary sequence (typically 10 bases long) are used to randomly amplify different segments of the genome (*16*, *17*). Recent attempts to study genetic diversity and understand the population genetic structure of some *Echinochloa* species have utilized isozyme and randomly amplified polymorphic RAPD markers (*4*, *10*, *14*, *18*, *19*), and these studies have shed some light on the taxonomic relations of *Echinochloa* spp. The origin of resistant (*19*) populations of *E. crus-galli* has also been examined using RAPDs, whereas RAPD, rDNA, and cpDNA markers were used to investigate the origins of cold-adapted populations of this species (*18*).

The specific objectives of the present study were to (a) perform dose−response studies to CB in populations of different species of *Echinochloa* and a cultivar and a weedy population of rice (*O. sativa*) and (b) carry out morphometric and RAPD analyses in seven populations of *Echinochloa* distributed worldwide to assess the genetic variability at species level and provide information on the phylogenetic relationships between *Echinochloa* and *Oryza sativa* populations.

MATERIALS AND METHODS

Plant Material and Growing Conditions. Different *Echinochloa* species originating from different geographical locations were supplied by Herbiseed (www.herbiseed.com.) (**Table 1**), all of them with no records of CB applications. Seeds were germinated in Petri dishes containing 2 g L^{-1} KNO_3 solution. Seedlings were planted in pots (five plants per pot) containing peat and sandy loam potting mixture (1:2 v/v) in a growth chamber at 28/18 °C (day/night) in a 16 h photoperiod under 350 μmol m^{-2} s^{-1} photosynthetic photon-flux density and 80% relative humidity.

Dose−Response Assays. Dose−response experiments were conducted in the growth chamber in seven populations of *Echinochloa* and a cultivar (Bahia) and a weedy population of rice (*O. sativa*). Treatments were applied to plants at the 3−4 leaf stage, using a laboratory track sprayer equipped with a Tee-Jet 80.02E VS flat-fan nozzle delivering a spray volume of 300 L ha^{-1} at 200 kPa (*20*). CB was applied at the rates of 0, 20, 40, 60, 80, 100, 150, and 200 g of ai ha^{-1} for all of the *Echinochloa* species and at 0, 300, 600, 1200, and 1800 g of ai ha^{-1} for the cultivar and the weedy population of rice. Above-ground fresh weight per pot was determined 21 days after spraying, and data were expressed as percentage of the untreated control. Herbicide rates to inhibit plant growth by 50% decrease in growth with respect to the untreated control (ED_{50}) were determined, and the *R*/*S* ratio was computed as ED_{50} (*R*)/ED_{50} (*S*) (*20*). Treatments were replicated three times and were arranged in a completely randomized design with four replications per dose. Data were pooled and fitted to a nonlinear, log−logistic regression model

$$Y = c + \{(d - c)/[1 + (x/g)^b]\}$$

where *Y* is the fresh above-ground weight expressed as percentage of the untreated control, *c* and *d* are the coefficients corresponding to the lower and upper asymptotes, *b* is the slope of the line, *g* is the herbicide rate at the point of inflection halfway between the upper and lower asymptotes, and *x* (independent variable) is the herbicide dose. Regression analysis was conducted using the Graphpad Prism 3.03 statistical software (*21*, *22*).

Morphometric Analyses. A morphometric analysis was carried out on 17−30 spikelets, including seeds (caryopses), and on 8−15 1-week-old seedlings from each of the *Echinochloa* spp. and *O. sativa* populations. Examined characters on spikelets were length, width, thickness, shape (see below), length of the lower and upper glumes, lemma length of the lower (sterile) and upper florets, setae length, and presence/absence of awns in glumes and lemmas. Awns were treated as a qualitative character because awn length in *Echinochloa* seems to be heavily dependent on environment, for example, the amount of moisture available. For a given population, the awnless character was assigned only if all of the spikelets examined had unawned glumes or lemmas. Moreover, the characters used on seeds were length, width, thickness, shape, hilum diameter, and external mark (length) of the embryo. Characters measured on seedlings were coleoptile length, blade length and width of the first leaf, and length-to-width ratio of the first leaf. The shape of spikelets and seeds was perceived, according to ref *23*, as the variance of their three dimensions, each divided by length so that the length was unity, $\Sigma (x - \bar{x})^2/3$. In this way, the shape becomes dimensionless and can vary between 0 (spherical) and 0.2 (disk- or needle-shaped).

DNA Extraction. Genomic DNA was extracted from the youngest leaves of each sampled plant. The extraction procedure was performed according to the manufacturer's recommendations (DNeasy plant mini kit, Qiagen GmbH, Hilden, Germany). The DNA content and quality of extraction was checked by gel electrophoresis.

PCR Amplification. A single PCR consisted of ca. 25 ng of DNA template, 12.5 μM of each primer, 1.25 mM of each dNTP (PCR nucleotide mix, Roche Diagnostic GmbH, Mannheim, Germany), and 1.25 U of Taq DNA polymerase (Roche Diagnostics) with 1× concentration of the supplied buffer in a final volume of 25 μl. The reactions were cycled on a thermocycler (Touchgene Gradient, Techne, Duxford, Cambridge, U.K.) with 35 cycles of 92 °C (DNA denaturation) for 30 s, 35 °C (primer annealing) for 30 s, and 72 °C (primer elongation by polymerase) for 30 s. Approximately 60 random decamer primers of kits OP-A, -B, and -R (Qiagen Operon Technologies Inc., Alameda, CA) were screened for their suitability for the generation of reproducible DNA profiles. Aliquots of the PCR products were analyzed by gel electrophoresis, and fragment sizes were determined using a 100 bp ladder (GeneRuler, MBI Fermentas GmbH, St. Leon-Rot, Germany).

Statistical Analyses. Means and standard errors (SE) were computed for all quantitative morphological characters, and means were tested for group differences and compared using an analysis of variance (ANOVA) and a Tukey HSD post hoc test. Univariate statistical analyses were made using SPSS 9.0. In addition, two ordination methods were used, principal component analysis (PCA) and hierarchical clustering. PCA was performed using Statistica 5.1 by including the 15 quantitative characters measured in each spikelet from the 7 *Echinochloa* spp. populations. Spikelets with incomplete data were excluded from the analysis. Hierarchical clustering was performed using SPSS 9.0 on mean values of the 19 quantitative characters and on the 2 qualitative characters studied in both the *Echinochloa* spp. and *O. sativa* populations. To give the same weight to all of the characters, mean values of each quantitative character were standardized to the maximum, the maximum being the same. In turn, this approach allowed the inclusion of the qualitative characters of absence (0) or possible presence (1) of an awn in upper glumes and lower lemmas. The dendrogram was constructed on the basis of the average linkage between groups, using the Euclidean distance as a similarity index. For the RAPD data, a hierarchical clustering was also performed using NTSYS-pc software. Binary data matrices were constructed by scoring the amplified bands as present (+) for shared DNA and absent (−) when the corresponding fragments were either absent or very light in intensity using Jaccard's coefficient (*24*), which allowed us to calculate the similarity levels (%): $J(x,y) = a/(a + b + c)$, where *a* is the number of bands common to species *x* and *y* and *b* and *c* are the number of

Table 2. Comparison of Quantitative and Qualitative Characters from Spikelets and Seedlings for Seven *Echinochloa* Species and Two *O. sativa* Populations[a]

	E. crus-galli 6			*E. crus-galli 7*			*E. oryzicola*			*E. colona*			*E. crus-pavonis*			*E. utilis*			*E. muricata*			*O. sativa* cv. Bahía			*O. sativa* weed		
character	mean	SE	*N*	mean	SE	*N*	mean	SE	*N*	mean	SE	*N*	mean	SE	*N*	mean	SE	*N*	mean	SE	*N*	mean	SE	*N*	mean	SE	*N*
spikelets																												
spikelet length	3.43c	0.06	30	3.04bc	0.05	30	4.30d	0.07	30	2.27a	0.02	30	3.14bc	0.07	30	3.03b	0.05	30	3.05b	0.04	30	8.97f	0.08	30	8.12e	0.14	30
spikelet width	1.90c	0.03	29	1.65b	0.02	30	2.30e	0.03	30	1.28a	0.02	30	1.40a	0.02	30	2.07d	0.03	30	1.61b	0.03	29	2.45f	0.02	30	3.16g	0.04	30
spikelet thickness	1.49h	0.03	30	1.34g	0.04	28	1.93d	0.03	29	1.08a	0.02	30	1.21b	0.02	28	1.75c	0.03	28	1.11ab	0.02	29	2.04e	0.01	30	2.20f	0.02	29
spikelet shape[b]	0.06b	<0.01	29	0.06b	<0.01	28	0.06b	<0.01	29	0.05b	<0.01	29	0.08c	<0.01	30	0.03a	<0.01	30	0.07c	<0.01	29	0.13e	<0.01	29	0.10d	<0.01	30
lower glume length	1.49b	0.03	29	1.28ab	0.03	30	2.08c	0.07	30	1.10a	0.02	30	1.29ab	0.02	30	1.13a	0.03	30	1.24ab	0.03	27	2.34c	0.07	23	2.84d	0.17	30
upper glume length	3.19c	0.06	28	2.93bc	0.06	27	4.09d	0.08	30	2.17a	0.03	29	3.14c	0.06	29	2.72b	0.03	30	2.87bc	0.05	29	2.37a	0.08	25	2.82b	0.15	30
lower lemma length	3.19c	0.06	30	2.87b	0.05	29	4.02d	0.06	30	2.08a	0.03	30	2.84b	0.05	30	2.86b	0.04	29	2.90bc	0.05	29	8.33f	0.07	30	7.31e	0.15	29
upper lemma length	2.84b	0.05	27	2.62b	0.04	30	3.89c	0.06	30	1.90a	0.02	29	2.59b	0.04	30	2.84b	0.05	30	2.76b	0.03	29	7.87e	0.06	29	7.09d	0.16	30
setae length	0.64d	0.03	30	0.61d	0.03	27	0.41cd	0.04	29	0.31bc	0.01	29	0.51d	0.02	29	0.27b	0.02	28	0.54d	0.03	28	glabrousa			0.79e	0.06	30
upper glume awned	possible			possible			possible			no			possible			possible			possible			no			no		
lower lemma awned	possible			possible			possible			no			possible			possible			possible			no			possible		
seed length	1.84cd	0.02	27	1.80cd	0.02	23	2.54e	0.04	28	1.22a	0.03	21	1.63bc	0.02	23	2.02d	0.02	29	1.51ab	0.02	17	6.90g	0.07	30	5.82f	0.14	30
seed width	1.48d	0.02	26	1.33c	0.02	20	1.99f	0.03	27	0.94a	0.02	21	1.15b	0.02	23	1.77e	0.03	28	1.19b	0.03	19	2.12g	0.02	30	2.66h	0.04	30
seed thickness	0.86d	0.02	26	0.77cd	0.02	23	1.35f	0.03	28	0.56a	0.02	21	0.70bc	0.02	24	1.26e	0.03	29	0.60ab	0.02	20	1.79g	0.02	30	1.91h	0.02	30
seed shape[b]	0.05c	0.01	26	0.05c	<0.01	20	0.04b	<0.01	27	0.05c	<0.01	21	0.05c	<0.01	23	0.03a	<0.01	28	0.06c	<0.01	17	0.11e	<0.01	30	0.08d	<0.01	30
hilum diameter	0.38bc	0.01	25	0.40c	0.02	18	0.53e	0.01	28	0.25a	0.01	21	0.40c	0.02	22	0.46d	0.01	29	0.28a	0.01	17	0.34b	0.01	29	0.39c	0.01	29
length embryo	1.53d	0.02	27	1.37c	0.03	30	2.27g	0.03	28	0.95a	0.03	19	1.25bc	0.05	22	1.73e	0.02	28	1.18b	0.03	27	1.91f	0.03	30	1.72e	0.06	30
seedlings																												
coleoptile length	7.73de	0.22	15	7.91e	0.24	15	10.20f	0.45	15	3.34a	0.21	15	5.24b	0.17	15	8.27e	0.38	15	5.83bc	0.27	15	4.97b	0.23	15	6.55cd	0.37	15
blade length of first leaf	20.73e	1.04	15	20.70e	1.24	10	19.07e	1.41	15	7.91b	0.43	15	13.80cd	0.85	15	17.4de	1.69	15	9.75bc	0.84	8	0.91a	0.09	15	0.95a	0.09	15
blade width of first leaf	2.56cd	0.07	15	2.35c	0.05	15	1.67b	0.07	15	2.28c	0.12	15	1.68b	0.04	15	2.82d	0.08	15	1.55b	0.05	15	0.62a	0.03	15	0.44a	0.02	15
blade length/width first leaf	8.23b	0.53	15	8.82b	0.56	10	11.60c	0.93	15	3.32a	0.24	15	8.43b	0.45	15	6.24b	0.56	15	6.33b	0.46	8	1.43a	0.10	15	2.13a	0.14	15

[a] Mean, standard error (SE), and sample size (*N*) are given. All dimensional variables are given in mm. For each quantitative character, mean values with the same letters do not differ significantly according to a Tukey HSD test ($p < 0.05$). [b] Variance of the three dimensions, each divided by length. See text.

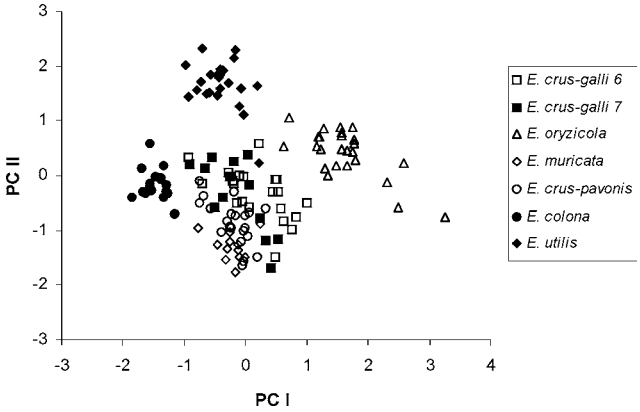

Figure 1. PCA based on 15 quantitative characters from spikelets of 7 *Echinochloa* populations.

bands for species *x* and *y*, respectively. The similarity data were analyzed using a group-average method, unweighted pair-group method with arithmetical averages (UPGMA) to create dendrograms providing a visual representation of the similarity matrices.

RESULTS AND DISCUSSION

Morphometric Analyses. Examination of individual quantitative characters of spikelets showed that *E. oryzicola* and *E. colona* populations both differed from the remaining five populations of *Echinochloa* by their overall larger and shorter size, respectively (**Table 2**). *E. oryzicola* differed significantly from all other *Echinochloa* populations with respect to all dimensional variables from spikelets, with the exception of setae length, and with respect to several seedling variables (**Table 2**). The same was true for *E. colona*, with the exception of spikelet and seed thickness, lower glume length, and hilum diameter (**Table 2**). The population of *E. colona* was also distinctive for its unawned upper glumes and lower lemmas (**Table 2**). The population of *E. utilis* differed significantly from all other populations in most of the characters examined in spikelets. *E. utilis* showed wider and thicker spikelets and seeds and, thus, a more spherical shape of both reproductive organs (**Table 2**). The remaining four populations of *Echinochloa* had no, or only a small number of, single quantitative characters. The two populations of *E. crus-galli* differed significantly in 5 of the 21 characters analyzed (**Table 2**).

The PCA of 15 quantitative characters of spikelets among the 7 *Echinochloa* populations clearly separated *E. oryzicola*, *E. colona*, and *E. utilis* but showed a large degree of overlap among the remaining populations (**Figure 1**). The PCA accounted for 85.35% of the total variance in the first two components, 67.16 and 18.19%, respectively. All dimensional characters, with the exception of setae length, were highly correlated with the first component. The second component axis emphasized the shape of both spikelets and seeds and setae length. The consistent larger and shorter sizes of *E. oryzicola* and *E. colona*, respectively, explain their separation along the first component, whereas the distinctive more spherical spikelets and seeds of *E. utilis* mainly explain their separation along the second component (**Figure 1**).

The hierarchical cluster analysis based on mean values of 19 quantitative characters and 2 qualitative characters clearly separated *Echinochloa* spp. from *O. sativa* populations (**Figure 2**). Among *Echinochloa*, the two *E. crus-galli* populations showed the greatest similarity, 96%, and *E. muricata* was closely related to *E. crus-pavonis*, clustering at 84% similarity. These two groups clustered at 73% similarity (**Figure 2**). The population of *E. oryzicola* showed a greater morphological affinity with the previous four populations than with *E. utilis* or *E. colona*, but linking only at 56% similarity (**Figure 2**). *E. utilis* and *E. colona* clustered at 56% similarity and formed a distinctive group among the *Echinochloa* populations, showing only 36% similarity with the cluster linking the remaining five populations (**Figure 2**). These results are in disagreement with cytogenetic (*25*, *26*) and molecular (*27*) data, suggesting that *E. utilis* is a domesticated derivative of *E. crus-galli*. However, crop domestication is an evolutionary process operating under manmade selection pressures, which usually result in marked morphological differences between domesticated and genetically related wild species (*28*).

Herbicide Treatment. Although all *Echinochloa* populations were controlled at the full field rate of CB (300 g of ai ha^{-1}), differences in susceptibility were found. *E. colona* was very susceptible (ED_{50} = 34 g of ai ha^{-1}) (**Table 3**), obtaining very similar results with the remaining populations with ED_{50} values ranging from 54 to 86 g of ai ha^{-1}. By contrast, *E. oryzicola* (170 g of ai ha^{-1}) was less sensitive than the rest of populations, with the herbicide symptoms appearing later. ED_{50} values (>1800 g of ai ha^{-1}) for the two *O. sativa* populations (**Table 3**) verify the selectivity profile of CB because of the capacity of rice to inactivate the esterase's functionality and metabolize CB to inactive polar metabolites (*29*). The effect on treated plants was rapid, with the first symptoms of phytotoxicity appearing 5 days after treatment. These symptoms were a visible reduction of plant growth together with chlorotic spots on the leaves. At higher herbicide rates, plants displayed a total growth stop, followed by a rapid plant degeneration leading to death within 15 days after treatment.

DNA Analyses. Among the 60 primers (OP-A, OP-B, and OP-R) screened, 21 primers were polymorphic and were thus selected on the basis of good banding patterns that could be scored readily (**Table 4**). RAPD primers used were OP-A (1, 4, 8, 11−14, 17, and 19), OP-B (2−4, 7, 10, 12, and 20), and

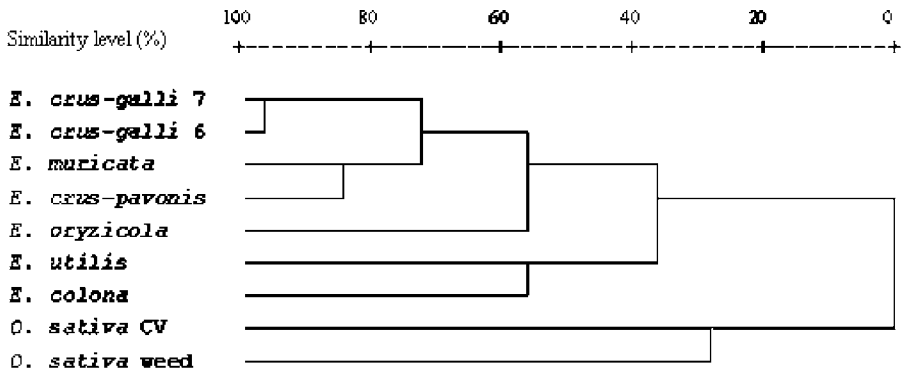

Figure 2. Dendrogram for *Echinochloa* spp. and *O. sativa* populations generated by hierarchical cluster analysis based on 21 morphological characters.

Table 3. Parameters of the Sigmoidal Equation[a] Fitted by Nonlinear Regression To Calculate the Herbicide Rates Required for 50% Reduction of Above-ground Fresh Weight (ED_{50}, Parameter *g*) of Different Populations of *Echinochloa* spp. and a Cultivar and Weedy Rice (*O. sativa*) from Dose–Response Experiments

population	*d*	*c*	*b*	ED_{50} (g of ai ha^{-1})	RMS[b]	pseudo r^2 [c]	*P*	*R*/*S*[d]
E. colona	98.56	3.68	3.86	34	43.12	0.9838	<0.0001	
E. crus-pavonis	100	4	4.14	54	35.93	0.9849	<0.0001	1.6
E. crus-galli 6	100	10	3.24	60	39.51	0.9821	<0.0001	1.76
E. utilis	100	6	2.67	62	41.33	0.9436	<0.0001	1.82
E. crus-galli 7	100	7	2.27	76	33.27	0.9921	<0.0001	2.24
E. muricata	100	15	1.54	86	34.59	0.9876	<0.0001	2.53
E. oryzicola	100	4.52	4.43	170	45.62	0.9710	<0.0001	5
O. sativa cv. Bahía				>1800				>53
O. sativa weed				>1800				>53

[a] $Y = c + \{(d - c)/[1 + (x/g)^b]\}$, where *Y* is the fresh above-ground weight expressed as percentage of the untreated control, *c* and *d* are the coefficients corresponding to the lower and upper asymptotes, *b* is the slope of the line, *g* is the herbicide rate at the point of inflection halfway between the upper and lower asymptotes, and *x* (independent variable) is the herbicide dose. [b] Residual mean square. [c] Approximate coefficient of determination for nonlinear models with a defined intercept calculated as pseudo $r^2 = 1 -$ (sums of squares of the regression/corrected total sums of squares). [d] *R*/*S* ratio was computed as ED_{50} (*R*)/ED_{50} (*S*).

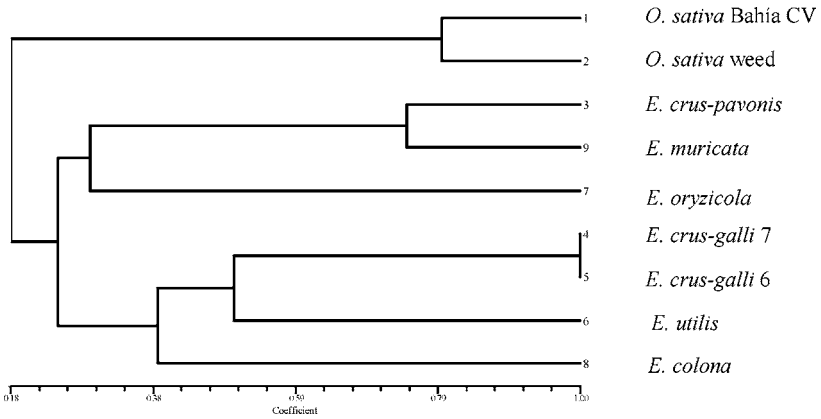

Figure 3. Dendrogram constructed from unweighted pair-group method (UPGMA) associations of rice and weedy rice (*O. sativa*) and seven populations of *Echinochloa* spp. based on analysis of the RAPD markers data.

Table 4. Selected Primers and Their Sequence and Level of Polymorphism

primer	sequence (5′–3′)	total no. of bands	no. of polymorphic bands	% polymorphism
OPA-01	CAGGCCCTTC	9	7	77.7
OPA-04	AATCGGGCTG	7	6	85.7
OPA-08	GTGACGTAGG	6	4	66.7
OPA-11	CAATCGCCGT	7	5	71.4
OPA-12	TCGGCGATAG	3	1	33.3
OPA-13	CAGCACCCAC	7	4	57.1
OPA-14	TCTGTGCTGG	7	7	100
OPA-17	GACCGCTTGT	6	3	50
OPA-19	CAAACGTCGG	7	5	71.4
OPB-02	TGATCCCTGG	9	6	66.7
OPB-03	CATCCCCCTG	8	5	62.5
OPB-04	GGACTGGAGT	3	2	66.7
OPB-07	GGTGAGCGAG	6	4	66.6
OPB-10	CTGCTGGGAC	7	4	57.1
OPB-12	CCTTGACGCA	9	5	55.5
OPB-20	GGACCCTTAC	8	5	62.5
OPR-02	CACAGCTGCC	4	2	50
OPR-03	ACACAGAGGG	5	3	60
OPR-10	CCATTCCCCA	7	5	71.4
OPR-16	CTCTGCGCGT	8	6	75
OPR-20	ACGGCAAGGA	3	1	33.3
total		136	90	63.8

OP-R (2, 3, 10, 16, and 20) (*4*) (**Table 4**). The 21 primers produced a total of 136 RAPD markers with a maximum of 9 bands in OPA-01, OPB-02, OPB-12 and the lowest score of fragments amplified, that is, 3 in OPA-12, OPB-04, and OPR-20 (**Table 4**). The rest of the primers were invariable, that is, present in the populations examined, and these RAPD markers may be genomic markers for this group of populations (*27*). Of all of the amplified fragments, 90 were found to be polymorphic. However, OPA-14 was found to produce 100% polymorphic fragments. The lowest polymorphism (33.3%) was seen in primers OPA-12 and OPR-20 (**Table 4**).

The dendrogram clearly separated *O. sativa* from *Echinochloa* spp. populations (18% similarity, **Figure 3**). At the morphological level, the two rice populations exhibited a lesser similarity (**Figure 2**) compared to the molecular level (**Figure 3**). The genetic diversity of weedy rice has been reported using AFLP methodology (*30*), and abundant polymorphisms were found among the populations tested. Due to the genetic similarity obtained, the weedy rice is presumed to most closely mimic cultivated rice and may have recently evolved. This suggests that weedy rice could adapt either to the natural environment or to cultivation (*30*).

The resolution of the seven *Echinochloa* spp. populations at the molecular level was fairly consistent with the results of the morphological analysis. The RAPD analyses showed a maximum level of similarity (100%) between *E. crus-galli* 6 and *E. crus-galli* 7. The *E. utilis* population appeared as being separated from the *E. crus-galli* 6−*E. crus-galli* 7 group but clustered at 52% similarity (**Figure 3**). This result is in agreement with the view that *E. crus-galli* is the direct ancestor of *E. utilis*, both hexaploid annuals (*25*−*27*). The populations that were morphologically different to the extent of being included in different species proved to be genetically closely related. The existence

of numerous intergrading races has been pointed out in *E. crus-galli* (*31*). *E. oryzicola* and *E. colona* were clearly distinctive among the *Echinochloa* populations at the molecular level, clustering at 30 and 38% similarity with the *E. crus-pavonis*−*E. muricata* group and the *E. utilis*−*E. crus-galli* 6−*E. crus-galli* 7 group, respectively (**Figure 3**). No significant differences were found with respect to sensitivity to CB, indicating that there was no relationship between these populations and their behavior toward the herbicide, although some authors do suggest a relationship between populations of *Echinochloa* and herbicide behavior. Thus, according to ref *4*, *E. colona*, *E. oryzoides*, and *E. oryzicola* are very susceptible to quinclorac treatments in Spanish paddy fields; by contrast, *E. crus-galli* and *E. hispidula* show some degree of natural tolerance (*4*).

A high degree of variability in RAPD markers was observed in *E. utilis* (data not shown), this being parallel to the high degree of morphological variability observed in this species. This variability could be due to possible multiple domestication events for the crop in different regions and the subsequent gene flow between the different cultivars. The genetic variability in *E. utilis* is particularly high when one considers the great inbreeding nature of the crop. On the other hand, the low genetic variability in crops, such as that found in barnyard millet, is not surprising (*32*). Crop domestication is a relatively recent (about 10000 years) evolutionary process from a few wild populations. Consequently, crop populations constitute a subset of the variability in wild ancestral species. Mutations, crosses between genetically diverged cultivars, and a possible gene flow with the wild and weedy progenitor contribute to their genetic diversity.

The existence of morphologically intergrading types is a well-known problem for taxonomy and species identification within *Echinochloa*. A univariate analysis showed that all of the quantitative characters examined differed significantly between one or more populations. Likewise, the ordination analyses exhibited a high degree of morphological overlap among most *Echinochloa* populations. In this sense, RAPD analyses, which were in general agreement with morphological results, can provide useful information about the genetic diversity and relationships among *Echinochloa* species. However, due to the simplicity of this technique, more accurate studies should be performed, for example, amplified fragment length polymorphism (AFLP) fingerprinting, which is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (*33*, *34*) and allows the generation of a larger number of polymorphisms. Compared with arbitrary-primed PCR, AFLPs are performed under high stringency and are therefore less sensitive to reaction conditions but produce a multilocus fingerprint with polymorphism being apparent as either band presence or band absence (*35*). AFLPs and microsatellites (SSRs) in *Echinochloa* have been used recently to assess variations in *E. crus-galli*, *E. colona*, and *E. crus-pavonis*, suggesting that these techniques may be useful for discriminating genotypes, studying population structure, and resolving taxonomic relationships in *Echinochloa* species (*31*).

ACKNOWLEDGMENT

We thank Dow Agrosciences S.A. for supplying chemicals. We are very grateful to Dr. Millán for statistical assistance and advice.

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Received for review August 23, 2005. Revised manuscript received November 29, 2005. Accepted December 2, 2005. We thank Project CO3-187 and AGL2004-8357 for supporting this research.

JF0520746