FOOD SCIENCE & TECHNOLOGY | REVIEW ARTICLE

Genetic variability of wild poinsettia populations in Brazilian agroecosystems

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Abstract: The use of molecular markers based on DNA sequences may be a promising strategy for assessing DNA-level genetic diversity in samples of wild poinsettia populations. Inter simple sequence repeat (ISSR) markers were used to assess the population structure and genetic diversity of 10 wild poinsettia populations sampled from soybean fields. A total of 133 DNA segments were amplified with nine ISSR primers, with an average of 14.77 amplicons per primer. ISSR markers showed high genetic polymorphism (98%) and divergence (Gst = 0.3607) among subpopulations. This study's results suggest that founding and bottleneck effects, as well as different selection pressures, adopted in wild poinsettia control selected different biotypes in each subpopulation and may have caused highly structured populations within species. This analysis of wild poinsettia at the DNA level was important to identify subpopulations with the highest and lowest genetic diversity and to underscore the need for different strategies to control populations in each infested field. Furthermore, ISSR markers may help to monitor the infestation dynamics of each cropping field.

Subjects: Environment & Agriculture; Biochemistry; Agriculture

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PUBLIC INTEREST STATEMENT

Weed plants that are commonly known as wild poinsettia in the USA and "amendoim bravo" or "leiteiro" in Brazil are the principal invasive species in soybean fields in Brazil. Infestations by wild poinsettia affect yield and agricultural productivity, causing large agricultural losses. The current study revealed that 10 wild poinsettia populations are genetically different. We suspect that the founding of populations by a small number of plants in a limited area determines the divergence among populations. Moreover, different selection pressures imposed by herbicides, either alone or in combination with various chemical groups, or herbicides with different mechanisms of action may also have caused the selection of different plants and the formation of different wild poinsettia populations. Differences among wild poinsettia populations are important because they indicate different capacities of the populations to invade new crop fields and indicate the need for different strategies to control populations in each infested field.

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1. Introduction

The invasive species *Euphorbia heterophylla* L. (Euphorbiaceae) is an herbaceous plant of economic relevance due to its invasive potential, causing losses to agriculture and pastures (Lorenzi, 2000). On the one hand, this species has potential applications as a medicinal plant (Vamsidhar, Mohammed, Nataraj, Rao, & Mullangi, 2000) and as a feed supplement for animals (Kouakou et al., 2013). *E. heterophylla* is native to the tropical and subtropical regions of the Americas (Barreto & Evans, 1998); this species, commonly known as wild poinsettia in the USA and "amendoim bravo" or "leiteiro" in Brazil, is one of the principal invasive weeds in soybean fields in Brazil (*Glycine max* Merr.) (Lorenzi, 2000; Trezzi et al., 2009).

Le Roux and Wieczorek (2008) highlighted the contribution of molecular methods to assess the genetic variability or diversity of populations in different species and to obtain in-depth knowledge on the management of invasive species. Isozyme analysis has shown high genetic diversity within local populations and high genetic diversity among populations of wild poinsettia (Frigo, Mangolin, Oliveira, & Machado, 2009). However, protein electrophoresis has also revealed only a small fraction of genetic variation in a population. This lack of variance is observed because polymorphisms in the genome's noncoding regions are not detected. Non-neutrality and a trend toward monomorphism, despite DNA sequence variation, are expected in studies on protein polymorphisms due to silent mutations.

The use of molecular markers based on DNA sequences may be a promising strategy for assessing DNA-level genetic variability in samples of wild poinsettia populations. Inter simple sequence repeat (ISSR) analysis may represent a feasible approach. ISSRs are DNA sequences of 100 to 3000 bp amplified by PCR using a single primer (16–20 bp) from microsatellite sequences (Gupta et al., 1994). Because ISSRs may be employed to infer relationships among closely related taxa (Wolfe, Xiang, & Kephart, 1998), ISSR markers were used in the current study to assess the polymorphism and genetic variability of samples from 10 wild poinsettia populations collected from soybean fields in southern Brazil. The authors' hypothesis is that the genetic polymorphism of ISSR markers in wild poinsettia is greater than the genetic polymorphism revealed by the study on isozymes. The authors suspect that the genetic variability of wild poinsettia at the DNA level is greater than the variability revealed by protein analysis. Assessing genetic variability may predict each invasive population's capacity to invade new crop fields and identify how populations of wild poinsettia are structured. Identifying the genetic structure of populations may help to establish strategies for the control of poinsettia populations in infested fields.

2. Materials and methods

Seeds of wild poinsettia were collected from invasive plants in soybean fields from 10 different localities in southeastern and southern Brazil: eight sites in the state of Paraná (PR) and one site each from the states of São Paulo (SP) and Rio Grande do Sul (RS) (Figure 1, Table 1). The populations were named according to the locations where they were collected (Table 1). Seeds were collected from 20 to 30 plants in each of the 10 different localities. Seeds from each site were distributed for germination in 500-mL pots containing sterile soil. Plants obtained from germinated seeds were maintained in a greenhouse (23°23′44.91″S; 51°57′3.13″W, altitude 510 m) at room temperature under daily irrigation.

Samples of young leaves were collected from plants 15 to 30 days after emergence. The samples used for DNA extraction were collected from 7 to 17 plants from each site for a total of 132 plants (Table 1). The number of plants obtained from the seeds varied and was influenced by the seed germination ratio from each site.

For DNA extraction, leaf sections (100 mg) from each plant were separately ground in liquid nitrogen and homogenized in a microcentrifuge tube with 800 μL extraction solution prepared with 100 mM Tris-HCl (pH 7.5)/50 mM EDTA (pH 8.0), supplemented with 5 M NaCl, 1% CTAB (cetyl trimethyl ammonium bromide), and 140 mM β-mercaptoethanol. After homogenization, the

Figure 1. Localities where the seeds of wild poinsettia were collected in the states of Paraná, São Paulo and Rio Grande do Sul, Brazil.

Table 1. *Euphorbia heterophylla* subpopulations sites, number of plants analyzed (NPA), and geographic location

Site (City—State)	NPA	Geographic location (UTM*)
PB-I (Pato Branco I, PR)	13	26°13'6.27" S/52°38'56.83" W
PB-II (Pato Branco II, PR)	17	26°11'43.83" S/52°43'14.49" W
COS (Cosmorama, SP)	13	23°26'53.73" S/52°15'30.73" W
MAR (Maringá, PR)	14	23°25'38" S/51°56'15" W
PB-III (Pato Branco III, PR)	07	26°17'2.78" S/52°39'48.13" W
IVA (Ivaiporã, PR)	16	24°14'26.14" S/51°36'16.58" W
SMI (São Miguel do Iguaçu, PR)	14	25°20'49.65" S/54°16'9.50" W
RIN (Rio dos Indios, RS)	11	25°20'49.65" S/54°16'9.50" W
JS-I (Jandaia do Sul I, PR)	15	23°36'11" S/51°38'36"W
JS-II (Jandaia do Sul II, PR)	12	25°38'22" S/53°40'38" W

*Universal Transversa de Mercator.

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Table 2. ISSR primers, nucleotide sequences of the ISSR primers, temperature, and number of primer-amplified segments in 132 wild poinsettia (*Euphorbia heterophylla*) samples			
Primer	**Sequency (5' → 3')**	**Temperature (ºC)**	**Amplified segments**
ISSR-2	$(AC)_8AG$	51°C	9
ISSR-6	$(AG)_8TA$	51°C	12
ISSR-7	$(AG)_8GA$	51°C	14
ISSR-11	$(AC)_8CA$	51°C	15
ISSR-12	$(AG)_8GCT$	52°C	21
ISSR-17	$(AG)_8CG$	51°C	19
lISSR-18	$(AG)_8TT$	50°C	16
ISSR-19	$(AG)_8TG$	50°C	13
ISSR-811	$(GA)_8C$	49°C	14
TOTAL			133

microcentrifuge tubes were gently shaken and incubated at 65°C for 60 min. DNA was extracted according to the Hoisington, Khairallah, and Gonzalez-de-Leon (1994) protocol. After DNA extraction, the samples were quantified using a UV-visible spectrophotometer Picodrop® (Pico 100–Version 4.0/21/03/11) to verify the DNA concentration of each sample before the DNA was diluted for PCR reactions.

2.1. Amplification reactions using inter simple sequence repeat primers

Nine ISSR primers (ISSR-2, ISSR-6, ISSR-7, ISSR-11, ISSR-12, ISSR-17, ISSR-18, ISSR-19, ISSR-811) selected for the current study were used with 132 DNA samples from wild poinsettia. DNA amplification with the nine primers was undertaken with an initial denaturation at 94ºC for 5 min and 35 cycles of 94ºC for 1 min, annealing for 1 min at 49 to 52ºC (Table 2) and final extension for 1 min and 30 s at 72ºC. Polymerase chain reaction (PCR) was performed with a Techne TC-512 thermal cycler. Amplifications were performed with 20 μL samples containing 15 ng genomic DNA; reaction buffer (10 mM Tris–HCl, pH 8.8); 2.4 mM $MgCl_2$; 0.1 mM each of dATP, dGTP, dCTP, and dTTP; 0.3 μM primer; and 1 unit of Invitrogen Platinum *Taq* DNA Polymerase and Milli-Q water to bring the reaction to the final volume. Electrophoresis was performed in a 1.5% agarose gel using 0.5 x TBE buffer (44.5 mM Tris-borate and 1 mM EDTA) at 60 V for 4 h. After electrophoresis, the gels were stained with ethidium bromide at 0.5 μg·mL^{-1}, and images were taken with a Molecular Image LOCCUS L-PIX—HE and Picasa 3 software. The size of the PCR fragments was determined with a 100-bp DNA ladder (Invitrogen).

Using POPGENE 1.32 software (Yeh, Yang, & Boyle, 1999), polymorphisms from ISSR loci were analyzed as dominant markers [(1) presence and (0) absence of amplified DNA segments] in the 10 wild poinsettia populations. The genetic diversity of Nei and genetic divergence represented by Nei's (1973) genetic differentiation were estimated in the 10 wild poinsettia populations. FreeTree software (Pavlícek, Hrdá, & Flegr, 1999) was also used to evaluate the genetic variability in samples of wild poinsettia. The genetic diversity of Nei and genetic divergence represented by Nei's (1973) genetic differentiation were estimated in bootstrap analyses for comparison among wild poinsettia plants. A distance similarity matrix was computed with UPGMA (Sneath & Sokal, 1973), followed by Jaccard's clustering method with resampling analysis, using 1000 replications. A dendrogram was constructed and drawn from a reference tree using the TreeView program (Page, 2001).

Analysis of molecular variance (AMOVA, GenAlEx 6.2; Peakall & Smouse, 2006) was performed to explore the hierarchical partitioning of genetic variation within and between wild poinsettia samples.

3. Results and discussion

Totally, 133 DNA segments were amplified with nine ISSR primers (ISSR-2, ISSR-6, ISSR-7, ISSR-11, ISSR-12, ISSR-17, ISSR-18, ISSR-19, ISSR-811), with an average of 14.77 amplicons per primer.

Since 131 segments were polymorphic, the percentage of polymorphism was 98% (Table 3). Our hypothesis that the genetic polymorphism at the DNA level in wild poinsettia is greater than the genetic polymorphism revealed by the study of isozymes was confirmed by ISSR markers. The genetic polymorphism of ISSR markers in samples of wild poinsettia populations reached 98%, while polymorphism in esterase loci was 87% (Frigo et al., 2009).

The size of the amplified products ranged between 300 and 2000 bp. Primer ISSR-12 generated the highest number of segments (21) and, therefore, the greatest capacity for discriminating polymorphic segments in the 10 wild poinsettia subpopulations. The highest polymorphism rate occurred in the PB-II subpopulation (88%), while the lowest rate was observed in the PB-I subpopulation (34%).

The genetic diversity of Nei (He) and the Shannon index varied between the samples and ranged from 0.1335 (PB-I) to 0.38 (PB-II) and from 0.1948 (PB-I) to 0.5462 (PB-II), respectively (Table 3).

The differential occurrence of ISSR segments observed in the subpopulations generated a high genetic divergence, represented by Nei's (1973) genetic differentiation (Gst = 0.3607). Consequently, the polymorphic DNA segments are different among the subpopulations. AMOVA showed higher genetic variation within (68%; sum of squares = 1725.869; variance components = 14.146) than among (32%; sum of squares = 927.748; variance components = 14.146) the 10 wild poinsettia subpopulations. ISSR markers revealed a relatively higher genetic differentiation level between the 10 wild poinsettia populations (G_{st} = 0.3607) than the population differentiation level observed with isozyme markers (F_{ST} = 0.1663) (Frigo et al., 2009).

The highest and lowest rates of genetic identity were detected between the JD-I and JD-II subpopulation (I = 0.942) and between the PB-I and PB-III subpopulations (I = 0.692), respectively. This fact revealed that the highest or lowest genetic diversity was not related to the geographic proximity between populations. A dendrogram generated by Jaccard's coefficient from the analysis of individual wild poinsettia plants (constructed from nine ISSR primers data using FreeTree software and the TreeView program) identified more than 10 well-defined groups formed by plants from 10 wild poinsettia subpopulations (Figure 2). The dendrogram showed several homogeneous groups formed by more than 80% of the plants from the COS (92%), PB-II (85%) and PB-I (84%) subpopulations. Other subpopulations formed small separate groups, and one plant from each PB-I, PB-II, COS and PB III-subpopulation formed isolated groups such that the 10 wild poinsettia subpopulations represented genetically divergent and well-structured genomes. In accordance with findings described for other invasive species (Le Roux & Wieczorek, 2008), ISSR markers had high genetic diversity and divergence among subpopulations of *E. heterophylla* growing in different areas in southern and southeastern Brazil. Genetic analysis of ISSR markers in other populations of invasive species also showed high genetic variation both within and among the populations (Gui, Wan, & Guo, 2009; Huangfu, Song, & Qiang, 2009; McRoberts, Sinclair, McPherson, & Marshall, 2005).
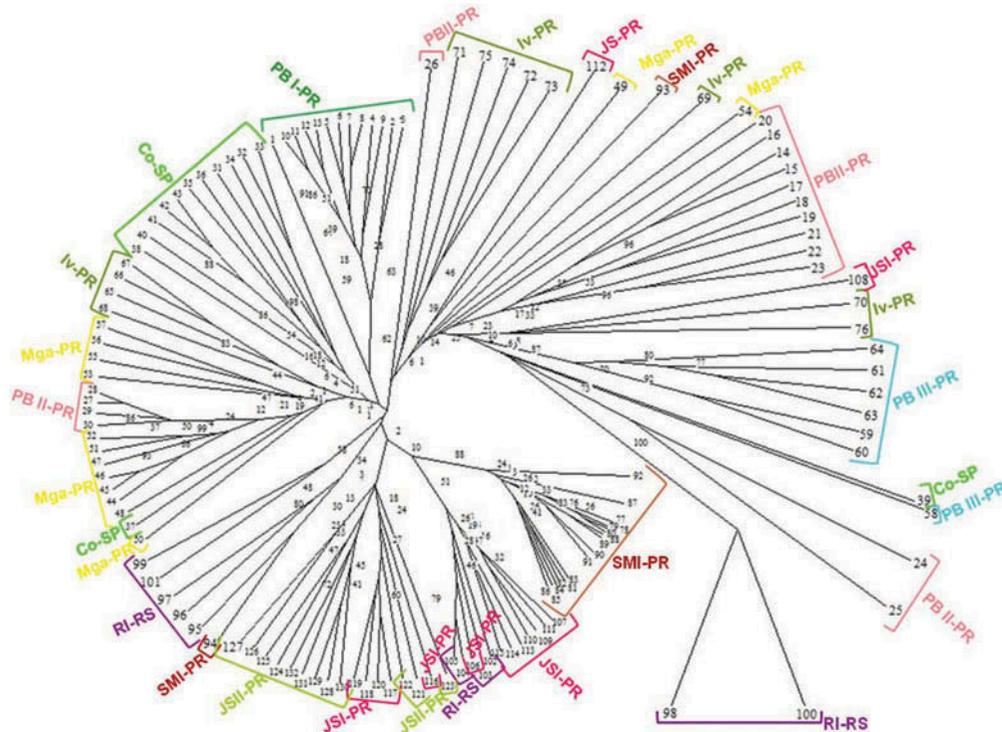
The highest polymorphism (88%) and genetic diversity (He = 0.38) detected in the PB-II subpopulation may account for the plants' greater colonization ability in other fields. Crawford and Whitney (2010) have shown that increased genetic variability enhanced colonization success, including seedling emergence rates, biomass production, flowering duration and reproduction, in experimental populations of *Arabidopsis thaliana*. The results are consistent with the premise that a high genetic diversity level would be an asset for the establishment of a population. The evidence that high genetic diversity contributed to increased colonization success in weeds was relevant for estimating the genetic diversity of wild poinsettia populations. As a control strategy, total extermination and avoiding the dispersal of wild poinsettia plants from populations with high genetic diversity should be performed.

In contrast, wild poinsettia populations with less genetic diversity may be a target for sources of phytochemical compounds. Uniform plant populations (exhibiting lower genetic variability levels) are suitable for carrying out extraction procedures because the same protocol may be quickly and easily standardized for most genetically uniform material. Plants from the PB-I population, e.g. the

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Table 3. Number of polymorphic segments (NPS), polymorphism (P%), genetic diversity of Nei (He) and Shannon index (I) in wild poinsettia subpopulations obtained from plants of Brazilian cities: Pato Branco (PB-I, PB-II, PB-III), Cosmorama (COS), Maringá (MAR), Ivaiporã (IVA), São Miguel do Iguaçu (SMI), Rio dos Índios (RIN), and Jandaia do Sul (JS-I and JS-II) in the States (Paraná, PR; São Paulo, SP and Rio Grande do Sul, RS)

Site (State)	PB-I (PR)	PB-II (PR)	COS (SP)	MAR (PR)	PB-III (PR)	IVA (PR)	SMI (PR)	RIN (RS)	JS-I (PR)	JS-II (PR)	Total
NPS	45	117	84	82	78	107	64	74	81	57	131
P (%)	34%	88%	63%	61%	58%	80%	48%	55%	60%	42%	98%
He	0.1335	0.3800	0.2466	0.2488	0.2225	0.3213	0.1708	0.2195	0.2172	0.1487	0.3589
I	0.1948	0.5462	0.3599	0.3608	0.3269	0.4671	0.2545	0.3202	0.3202	0.2222	0.5345

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Figure 2. Dendrogram generated using Jaccard coefficient from the analysis of individual plants from the wild poinsettia populations obtained from 10 different sites in the cities of Pato Branco (PB-I, PB-II, PB-III), Cosmorama (COS), Maringá (MAR), Ivaiporã (IVA), São Miguel do Iguaçu (SMI), Rio dos Índios (RIN), and Jandaia do Sul (JS-I and JS-II) in the States (Paraná, PR; São Paulo, SP and Rio Grande do Sul, RS) of Brazil based on 133 ISSR markers. Numbers next to the nodes indicate relative bootstrap frequencies (%).

population with the lowest polymorphism (34%) and genetic diversity (He = 0.1335), may be recommended as a source of material for extraction procedures. The antimicrobial properties of sections of *E. heterophylla* against different species of *Salmonella* have been reported (Enemaduku, Yahaya, Garba, & Samuel, 2011). The leaves of *E. heterophylla* have been used in traditional medical practices, and plant extracts have been identified as an economical and effective alternative treatment (Atarod, Nasrollahzadeh, & Sajadi, 2016).

The genetic divergence rate (Gst = 0.3607) in the current analysis is consistent with that for mixed-mating species (Hamrick & Godt, 1996). Wild poinsettia may be reproduced by self- or cross-fertilization (Barroso, 2002). The genetic variability and structure of populations in weeds is commonly affected by several evolutionary factors, including mating system, gene flow via pollen and seed dispersal, geographic range, and natural or directed selection. Genetically structured populations frequently result from founding and bottleneck effects or limited spatial dispersal. Furthermore, inbreeding usually reduces gene exchange (gene flow) between different individuals and different populations, leading to significant differentiation between populations.

The bottleneck effect and limited spatial dispersal have been frequently indicated as factors that determine the genetic structure of other weeds (Chase, 2011; Wang, Su, & Chen, 2008). We suspect that different selection pressures imposed by herbicides, either alone, in combination with different chemical groups, or with different mechanisms of action (Correia & Kronka, 2010; Oliveira et al., 2011; Prigol et al., 2014), may also have caused the selection of different biotypes and the formation of genetically structured wild poinsettia populations. Preliminary data from studies evaluating esterase isozymes also suggest that populations of *E. heterophylla* have been under high selection pressure imposed by herbicides (Frigo et al., 2009). Structural chromosomal rearrangements were reported in the genome of *E. heterophylla* and may also be attributed to the use of herbicides (Aarestrup, Karam, & Fernandes, 2008).

Selection of different biotypes and the formation of genetically structured wild poinsettia subpopulations may require different control strategies for each field. Consequently, different control

strategies for each field may lead to the formation of genetically structured wild poinsettia subpopulations. In this way, the control of wild poinsettia is increasingly difficult owing to the differentiated control strategies that lead to the formation of subpopulations with different predominant genomes.

Current analysis of wild poinsettia at the DNA level was important to i) identify subpopulations with the highest and lowest genetic diversity; ii) decipher the genetic structure of wild poinsettia populations and suppose that different mechanisms of action may have caused the formation of genetically structured wild poinsettia populations; iii) identify the need for different strategies for the control of populations in each infested field; and iv) suppose that the formation of subpopulations with a predominance of different genomes may also hinder control in addition to the selection of resistant biotypes. Furthermore, ISSR markers may help to monitor the infestation dynamics of each cropping field. With such a tool, it will be possible to verify whether there are alterations in the genome and the composition of prevalent groups in each field associated with an established control plan to respond to how the bottleneck effect directs the evolution of *E. heterophylla*.

4. Conclusions

In wild poinsettia populations in Brazilian agroecosystems, the genetic polymorphism based on DNA sequences is greater than the genetic polymorphism revealed by the study of isozymes. The genetic structure of populations is high, with a predominance of different genomes. Several evolutionary factors, as well as different selection pressures adopted in wild poinsettia control, seem to be selected for different biotypes in each subpopulation. The significant genetic differentiation observed among populations requires different control strategies for each field, causing the control of wild poinsettia to become increasingly difficult.

Key research activities
Our research group is named "Biochemical and Molecular Markers for Characterizing Genetic Diversity in Plants." The diversity and genetic divergence among different species and varieties of cacti, grapes, corn, sugarcane, cassava, peppers, and weeds using molecular markers have been investigated in our Laboratory of Biotechnology and Plant Genetics since the 1990s. The genetic diversity and structure of wild poinsettia populations, three species of the *Conyza* genus (hairy fleabane, horseweed, and fleabane), and sourgrass (*Digitaria insularis*) have been investigated since 2009 to establish strategies for the control of populations in infested fields.

Competing Interests
The authors declare no competing interests.

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