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Genetic diversity in *Veronica hederifolia* (Plantaginaceae), an invasive weed in China, assessed using AFLP markers

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Veronica hederifolia (Plantaginaceae) — an invasive, alien weed in eastern China — is of great concern due to its potential to spread. This study was conducted to determine the genetic diversity of *V. hederifolia* in comparison with several congeneric species, including *V. persica*, *V. arvensis, V. didyma* and *V. peregrina* as outgroups, using the Amplified Fragment Length Polymorphism (AFLP) technique. The samples of *V. hederifolia* were collected from 24 locations in Nanjing and Zhoushan, and genomic DNA was digested with the enzymes EcoRI/MseI and amplified with six E+3/M+3 primer combinations. Both mono- and polymorphic AFLP markers were analyzed to estimate genetic diversity of *V. hederifolia* based on a range of standard indicators. A high percentage of polymorphic bands (89.23%) existed in the populations examined, implying high genetic diversity. Based on all genetic indices, the Nanjing population, which was the first reported in China, exhibited greater genetic diversity than the Zhoushan population. Substantial variation was also found among individual plants collected from a single site near Nanjing, with 79.56% polymorphism. This high genetic diversity indicates this weed species potentially has broad adaptability. Cluster analysis of AFLP data placed *V. hederifolia* samples into three groups depending largely on geographical locations.

Key words: AFLP, alien invasive species, genetic diversity, Ivy-leaved speedwell, *Veronica hederifolia*

Introduction

Ivy-leaved speedwell (*Veronica hederifolia*), native to the Mediterranean region, is an invasive weed nearly worldwide. It can be a common weed in natural environments, and also cause crop yield losses as observed in many earlier invaded countries including UK, Germany, France, Poland, Austria, Bulgaria, Belgium and Italy (Roberts 1978, Cavero 2001, Milusheva 2002, Ozaslan 2002). In recently infested countries, such as Japan and Finland (Tsuruuchi 1971, Fenni 1993), *V. hederifolia* exhibited an exceptional ability to spread. In the 1980s, *V. hederifolia* was unintentionally introduced to China.

The infestation is presently scattered at two separate locations in two eastern provinces; a 20-km^2 area in Zhongshan Mountain Scenic Spot

in Nanjing City, Jiangsu Province, and a 1-km^2 area at the Zhoushan Martyrs Cemetery in Haishan park of Zhoushan Island, Zhejiang Province. The weed is most commonly seen along roadsides, in forest areas and public parterres in Nanjing (Wu & Qiang 2005). Although not yet found in crop fields, judging from its biology and infestation history in other parts of the world, *V. hederifolia* is likely capable of invading croplands in temperate and subtropical zones of China (Tsuruuchi 1971, Fenni 1993). The other related species within the same genus such as *V. persica*, *V. arvensis* and *V. didyma*, which share the same native range as *V. hederifolia*, have become common weeds in the temperate zone of China and cause serious problems for crop agriculture (Chen *et al*. 2001, Li 2003, Tan *et al*. 2004, Wu & Qiang 2004). Therefore, the potential for *V. hederifolia* to further spread and adapt in China is of great concern. A recent study showed that *V. hederifolia* had already reduced the biological diversity within the infested areas in China (Wu & Qiang 2005).

Genetic diversities and adaptive responses play a key role in the establishment and spread of many invasive species (Pappert *et al*. 2000, Lee 2002). The success of invasion can be facilitated by the presence of certain genetic traits in source populations upon which natural selection acts. Sufficient additive genetic variation is essential for evolutionary adaptation in response to environmental changes (Lee 2002). Molecular markers have been used in many studies to estimate genetic diversity of alien, invasive populations (Qian *et al*. 2000, Ash *et al*. 2003, Pester *et al*. 2003, Ye *et al*. 2003, 2004, Senda *et al*. 2004, 2005), and Amplified Fragment Length Polymorphism (AFLP) markers have been used successfully to determine genetic diversity, invasiveness and adaptation of several invasive weeds such as *Rubus alceifolius* (Amsellem *et al*. 2000), *Lolium temulentum* (Senda *et al*. 2005) and *Eupatorium adenophorum* (Duan *et al*. 2005). However no information is available on the genetic diversity of *V. hederifolia* populations in China.

The aim of our study was to determine the genetic diversity of *V. hederifolia* at different geographic locations in China in order to establish a baseline for assessment of the future dispersal and impact of this invasive species.

Material and methods

Samples of *Veronica hederifolia*

Leaf samples were collected from 24 sites in the two infestation areas in China: 21 in Nanjing (32°02–03´N, 118°49–50´E, alt. 8–115 m a.s.l.), Jiangsu Province; and 3 in Zhoushan (30°01´N, 122°05´E, alt. 32–36 m a.s.l.), just off the east coast of Zhejiang Province (Fig. 1). At each site, leaf samples (3–4 leaves per plant) were collected from ten randomly selected plants, following the protocol described by Gethi *et al*. (2005), to represent the plant genotype at the location. The distance between sampling sites in the same infestation areas was at least 0.5 km. Leaf samples of additional *Veronica* spp., including *V. persica*, *V. arvensis*, *V. didyma* and *V. peregrina*, were collected in the vicinity of Nanjing as outgroups following the similar protocol. To estimate genetic variation within sampling sites, 11 individual plants spaced 5 to 20 m were sampled at the Plum Blossom Hill site. About 2 g of leaf material was collected from each site and immediately stored in liquid nitrogen. The collection was carried out in spring when the majority of the plants were at the early-flower stage.

DNA isolation and PCR conditions

Genomic DNA was extracted from leaf samples following the procedure described by Doyle and Doyle (1987), and the DNA concentration was estimated and standardized against known concentrations of λDNA in 1.5% agarose gels using an UV spectrophotometer. Aliquots of DNA preparations were used for AFLP analyses (Vos *et al*. 1995, Veerle *et al*. 2005). 500 ng of genomic DNA was digested in a total volume of 15 μl at 37 °C for 6 h with 5 units (U) of EcoRI and MseI, respectively. The enzymes were inactivated at 94 °C for 5 min, and then cooled on ice. A 20-μl solution containing 300 pmol EcoRI adaptor, 300 pmol MseI adaptor, 0.6 U T4 DNA ligase in 2 μl of 10 × T4 DNA ligase buffer was prepared. Ligation of adaptors was performed for 2 h at 25 °C. Pre-selected amplification of restriction fragments was carried out with 4 μl of each DNA (20 ng), 1 μl EcoRI primer (50 ng),

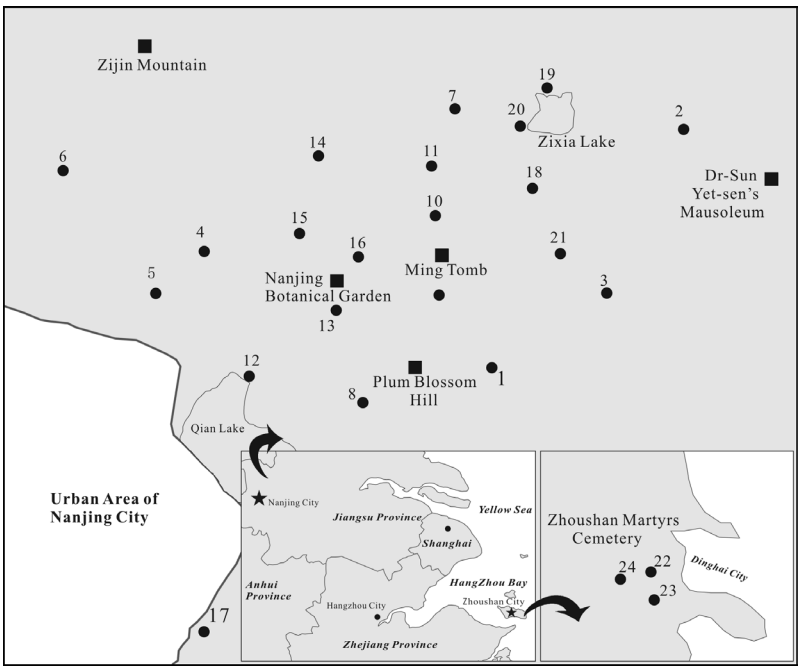

Fig. 1. Site locations for *Veronica hederifolia* samples collected near Nanjing and Zhoushan.

1 μl MseI primer (50 ng), 1.5 μl 10 × PCR buffer, 1.2 μl $MgCl_2$ (25mM), 1.2 μl DNTPs (2.5 mM), and 1 U of Taq DNA polymerase in a sequence for 20 cycles: 94 °C for 30 s, 56 °C for 60 s, and 72 °C for 60 s. Further selective amplification was carried out using 4 μl of each diluted DNA preparations (20 ng) using the same materials and protocol as for the pre-selective amplifications. In subsequent cycles, the annealing temperature was reduced by 0.7 °C per cycle until it reached 56 °C, followed by 23 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s.

Electrophoresis and silver staining

A 6% denaturing polyacrylamide gel was pre-run at 60 W for 90 min. Samples were denatured at 95 °C for 5 min and 8 μl aliquots were loaded per well. The electrophoresis was carried out in a BioRad Sequi-Gen GT DNA sequencing cell at 80 W and 48 °C for 3 h 20 min. After that, the gel was fixed in 1% ethanoic acid for 90 min, rinsed in distilled water and stained with a mixture of 0.2% silver nitrate and 0.007% benzene sulphonic acid for 30 min. The stained gel was rinsed again with distilled water and immersed in a developing solution containing 0.037% formaldehyde and 0.002% sodium thiosulphate. The development was subsequently stopped with 1% acetic acid when the bands were clearly visible.

Data analysis

Data were coded binary (1 = presence, 0 = absence) for each band level. Only markers that scored unequivocally were considered, and both mono- and polymorphic AFLP markers were used in data analyses. The resulting data matrix was analyzed using POPGENE ver. 1.32 (Yeh *et al*. 1999, Liu *et al*. 2005) to estimate genetic diversity parameters including observed numbers of alleles, the effective number of alleles per locus, the percentage of polymorphic loci (*P*), Shannon's information index (*I*) (Lewontin 1972), Nei's genetic diversity index (*H*) (Nei 1973), as well as unbiased genetic identity or distance between samples (Nei 1972). Based on the UPGMA method (Nei 1972, Schnell *et al*. 2001), using Jaccard's coefficient matrix (Jaccard 1908) a dendrogram was constructed with the aid of the software MVSP 3.131 (Kovach computing services, Wales, UK). An analysis of

molecular variance (AMOVA; Excoffier *et al.* 1992) of the program WINAMOVA 1.55 (http://anthropologie.unige.ch/ftp/comp) was used to partition the AFLP variation.

Results

Genetic diversity based on AFLP fingerprinting

AFLP fingerprinting of all 24 *V. hederifolia* sites revealed high levels of polymorphism (Table 1). A total of 492 bands were visualized using six pairs of primers. Of those, 439 were polymorphic, representing close to 90% of the total. An individual primer pair generated 64–108 bands, with 54–100 being polymorphic. The primer combination E-ACA/M-CAA produced the most numbers of scorable and polymorphic bands. The genotypes amplified with the six primer pairs matched the total number of *V. hederifolia* sites examined and no two sites were exactly the same.

Based on the AFLP analysis of *V. hederifolia* from the 24 sites, substantial genetic diversity was indicated by relatively high *H* and *I* values (Table 2). Other variability indicators, including the effective number of alleles per locus and genetic identities between samples also pointed to the same conclusion. The genetic distance among the 24 *V. hederifolia* sites ranged from 0.1072 to 0.4208 with a mean of 0.2257, while the genetic identities ranged from 0.6565 to 0.8984 with a mean of 0.7996. The lowest value (0.6565) of genetic identities for pair-wise comparisons was between the samples from Nanjing and Zhoushan.

Table 1. Polymorphism of *Veronica hederifolia* as revealed by six primer pairs.

Primer pair	Total bands	Polymorphic bands	Polymorphism (%)
E-AAC/M-CAA	64	60	93.75
E-AAC/M-CAT	66	54	81.82
E-AAC/M-CTA	103	90	87.38
E-ACA/M-CTA	69	61	88.41
E-ACA/M-CAT	82	74	90.24
E-ACA/M-CAA	108	100	92.59
Total	492	439	

When comparing the genetic diversity of *V. hederifolia* populations within the two areas, the Nanjing population showed slightly greater variability and a much higher percentage of polymorphism than the Zhoushan population (Table 2). In the AMOVA analysis, most of the total variance can be attributed to that within (76.3%) rather than between the populations (23.7%) of *V. hederifolia* (Table 3).

Using the same primer combinations, a total of 318 bands were detected from the 11 individual *V. hederifolia* plants collected from the Plum Blossom Hill location in Nanjing (Table 2). Of those bands, 253 were polymorphic (79.6%).

Table 2. Genetic variation statistics for the *Veronica hederifolia* samples. Na = observed number of alleles, *I* = Shannon's information index, *H* = Nei's genetic diversity index.

Source of variation	Na	*I*	*H*	Polymorphism (%)
24 samples	1.8923 ± 0.3103	0.2286 ± 0.1695	0.1276 ± 0.119	89.23
11 individual plants	1.7956 ± 0.4039	0.3079 ± 0.208	0.1879 ± 0.1443	79.56
Nanjing population	1.7988 ± 0.4013	0.2118 ± 0.1785	0.1192 ± 0.1222	79.88
Zhoushan population	1.2988 ± 0.4582	0.1682 ± 0.2638	0.1133 ± 0.181	29.88

Table 3. Analysis of genetic variation (AMOVA) for the *Veronica hederifolia* samples from Nanjing and Zhoushan, China.

Source of variation	d.f.	SSD	MSD	Variance component	Total	*P*
Between populations	1	121.03	121.04	14.28	23.68%	< 0.1
Within population	22	1013.05	46.05	46.05	76.32%	< 0.1

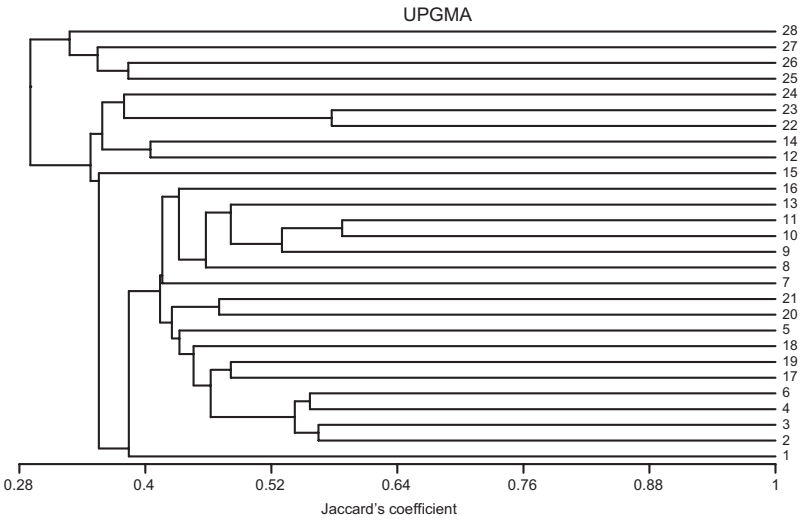

Fig. 2. UPGMA dendrogram of Jaccard's coefficient for 24 samples of *V. hederifolia* (1–21 from Nanjing population; 22–24 from Zhoushan population) and three samples of additional exotic species *V. persica*, *V. arvensis*, and *V. didyma* (25, 26, and 27), and the native species *V. peregrina* (28).

This level of polymorphism was similar to that observed within the Nanjing overall population. The AFLP analysis also indicated a relatively high genetic diversity among these nearby plants at this sampling location, and the variation was only slightly smaller than that observed in samples from different locations.

Cluster analysis

Dendrograms were constructed using Jaccard's coefficient of similarity based on AFLP data from 28 sites. Two major clusters were identified (Fig. 2): one consisting of *V. peregrina* (25), *V. persica* (26), *V. didyma* (27) and *V. arvensis* (28), and the other including the 24 *V. hederifolia* samples that were divided further into three sub-clusters. Among different species, the lowest genetic similarity was between *V. hederifolia* sample no. 5 and *V. persica* (0.264). *V. hederifolia* collected from Zhoushan (sites 22, 23, 24), with similarity values ranging from 0.42 to 0.708 (Table 4), appear together in the same sub-cluster. As compared with *V. hederifolia* from the Zhoushan sites, the plants from the Nanjing area were genetically more variable (similarity value 0.329–0.625) and could be further divided into two sub-clusters; plants collected from sites 12 and 14 in the Nanjing Botanical Garden were in one sub-cluster and the others that were collected outside the Botanical Garden belonged to the other. Plants from sites 12 and 14 were genetically closer to the Zhoushan population (Fig. 2).

Discussion

Genetic structure and diversity are the key elements in population biology (Sakai *et al*. 2001). Genetic diversity is highly linked to the adaptive capability and evolutionary potential of a species, and consequently it has a profound bearing on the distribution and population composition of a species (Lee 2002). Greater genetic diversity tends to allow better adaptation to a wider range of environmental conditions (Zou *et al*. 2001). With the spreading of an invasive species, multiple introductions and gene flow occur frequently and some variations are retained in the populations. Some of the changes can be more readily detected at molecular levels long before major physiological adaptation and morphological differentiation become evident. Gene flow within a plant population can occur through pollen and seeds (Ouborg *et al*. 1999). Hybridization of *V. hederifolia* with other *Veronica* species is a possibility due to the high rates of cross pollination and natural hybridization (Tsuruuchi 1994) and the coexistence of these species at many locations. Invasiveness of a species may depend on several traits but the rate of hybridization after initial colonization is critical to genetic diversity of the population (Chen *et al*. 2004) and

Table 4. Similarity matrix for 24 samples of *Veronica hederifolia* (1–21 from the Nanjing population; 22–24 from the Zhoushan population) and three samples of additional exotic species *V. persica*, *V. arvensis*, and *V. didyma* (25, 26, and 27), and the native species *V. peregrina* (28).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
2	0.411	–																									
3	0.449	0.587	–																								
4	0.500	0.570	0.619	–																							
5	0.484	0.503	0.471	0.511	–																						
6	0.486	0.567	0.572	0.617	0.494	–																					
7	0.411	0.531	0.459	0.497	0.500	0.455	–																				
8	0.426	0.488	0.486	0.434	0.478	0.411	0.532	–																			
9	0.390	0.535	0.467	0.494	0.517	0.463	0.545	0.555	–																		
10	0.360	0.531	0.465	0.491	0.418	0.461	0.530	0.533	0.584	–																	
11	0.350	0.540	0.416	0.497	0.489	0.447	0.551	0.467	0.608	0.625	–																
12	0.380	0.447	0.416	0.435	0.388	0.429	0.358	0.396	0.435	0.404	0.412	–															
13	0.459	0.503	0.519	0.583	0.493	0.559	0.460	0.453	0.497	0.494	0.510	0.503	–														
14	0.326	0.383	0.369	0.377	0.367	0.414	0.340	0.353	0.346	0.371	0.376	0.457	0.429	–													
15	0.397	0.439	0.414	0.426	0.434	0.450	0.337	0.372	0.358	0.386	0.393	0.384	0.497	0.404	–												
16	0.397	0.478	0.424	0.474	0.456	0.462	0.435	0.465	0.510	0.443	0.503	0.419	0.497	0.383	0.417	–											
17	0.458	0.575	0.519	0.531	0.526	0.531	0.449	0.452	0.487	0.447	0.480	0.422	0.524	0.365	0.470	0.497	–										
18	0.454	0.469	0.500	0.509	0.448	0.486	0.455	0.397	0.446	0.405	0.439	0.362	0.476	0.345	0.442	0.438	0.522	–									
19	0.446	0.536	0.490	0.568	0.590	0.503	0.523	0.468	0.540	0.493	0.557	0.385	0.493	0.409	0.432	0.529	0.574	0.554	–								
20	0.467	0.466	0.472	0.572	0.463	0.485	0.510	0.437	0.487	0.440	0.433	0.338	0.465	0.329	0.375	0.406	0.534	0.503	0.608	–							
21	0.392	0.529	0.479	0.557	0.470	0.449	0.467	0.443	0.457	0.509	0.468	0.358	0.453	0.354	0.382	0.430	0.500	0.474	0.555	0.560	–						
22	0.356	0.500	0.405	0.440	0.439	0.432	0.398	0.392	0.431	0.415	0.415	0.436	0.404	0.390	0.367	0.430	0.458	0.411	0.429	0.389	0.497	–					
23	0.305	0.419	0.379	0.388	0.392	0.368	0.340	0.340	0.388	0.369	0.361	0.444	0.359	0.375	0.331	0.362	0.389	0.362	0.376	0.335	0.416	0.708	–				
24	0.357	0.474	0.424	0.404	0.402	0.407	0.369	0.374	0.389	0.352	0.420	0.406	0.377	0.344	0.410	0.411	0.406	0.372	0.400	0.333	0.347	0.459	0.420	–			
25	0.275	0.326	0.320	0.319	0.340	0.282	0.288	0.294	0.309	0.309	0.330	0.371	0.329	0.350	0.307	0.325	0.306	0.293	0.344	0.298	0.335	0.404	0.438	0.400	–		
26	0.274	0.287	0.319	0.286	0.264	0.285	0.291	0.296	0.268	0.299	0.279	0.359	0.315	0.312	0.339	0.276	0.280	0.331	0.281	0.283	0.305	0.340	0.404	0.372	0.464	–	
27	0.287	0.295	0.316	0.320	0.279	0.337	0.270	0.267	0.307	0.297	0.303	0.354	0.328	0.319	0.345	0.305	0.269	0.317	0.286	0.297	0.293	0.345	0.341	0.356	0.421	0.439	–
28	0.302	0.347	0.347	0.327	0.380	0.316	0.314	0.324	0.348	0.309	0.339	0.344	0.330	0.302	0.290	0.361	0.326	0.310	0.346	0.323	0.350	0.403	0.379	0.372	0.395	0.337	0.394

long-term success of the species (Ellstrand & Schierenbeck 2000). Varying chromosome numbers may also reflect the genetic diversity of *V. hederifolia*. Guo and Liu (2001) reported that the chromosome numbers for *V. hederifolia* from the Nanjing area were 18, 22, 32, 36, or 54, with 18 and 32 being most common.

Percentage of polymorphic bands within a population gives a direct estimate of the level of genetic diversity (David *et al*. 2001). For an invasive species, lower genetic differentiation within a population may be due to a combination of reasons such as limited genetic drifting, founder effects, and environmental effects (Meekins *et al*. 2001). High genetic variability was shown in the *Veronica* species, which suggests the effectiveness of AFLP in differentiating these weeds at both intra- and inter-specific levels. This observation is consistent with those on several other invasive weed species (Amsellem *et al*. 2000, Duan *et al*. 2005, Senda *et al*. 2005). The level of genetic diversity in this study (P = 89.23%) is also comparable to that reported for other invasive weeds. The percentage polymorphic fragments of *Eupatorium adenophorum* was reported to be 77.01% (Duan *et al*. 2005). Using AFLP techniques, Gethi *et al*. (2005) found that the percentages of polymorphic fragments in *Striga hermonthica* and *S. asiatica* were 84% and 96.8%, respectively. It should be pointed out, however, that not all invasive weeds show high levels of genetic diversity, good examples being *Fallopia japonica* (Hollingsworth & Bailey 2000) and *Alternanthera philoxeroides* (Wang *et al*. 2005). These two species seem to propagate and spread well through clonal growth.

Cluster analysis separated the different *Veronica* species effectively. It also clearly differentiated *V. hederifolia* from the related species. *Veronica hederifolia* was in two groups based on samples from the two separate infestation areas: Nanjing and Zhoushan. Sites from Nanjing can be further divided into two sub-groups, which suggests that some differentiation might have already occurred since the introduction of the weed into the Nanjing area 24 years ago (Chen & Liu 1986). More substantial variation at the DNA level within the Nanjing population may imply greater gene flows between varying species/biotypes of *Veronica* in the area. The mode of invasion by *V. hederifolia* on the Zhoushan island was unclear despite the first report on the weed 15 years ago (Editing Group of Flora of Zhejiang Province 1993). Direct introduction from a foreign source is a possibility due to frequent docking of fishing ships from overseas.

For most of the invasive species, new populations often become established from only a few individuals from native habitats (Elton 1958), and an invasion to a new territory is associated with frequent-founder effects, which can initially lead to decreased genetic diversity within the population (Nei *et al*. 1975, Tsutsui *et al*. 2000, Sakai *et al*. 2001). With time, the dynamics of genetic structures in a plant population is driven largely by gene flow, environmental selection pressure, and genetic drift (Ward 2006). As shown in Table 3, genetic variability was consistently higher in *V. hederifolia* from Nanjing, potentially suggesting a greater ability by the Nanjing population to invade the habitats occupied by other plant species. Following the five-step theory of the invasion process for alien species (Williamson 1996), *V. hederifolia* population is likely at the expansion stage in Nanjing while still establishing itself in Zhoushan. As an invader, *V. hederifolia* appears to be extremely competitive in its new range. Based on the survey, it was the most dominant species at all sampling locations, covering about 80% of the area (data not shown) and out-competing other species including *Stellaria media* and *Galium aparine*.

This is the first report on using AFLP markers to evaluate genetic variability in natural populations of *V. hederifolia*. The relatively high genetic diversity found in the two separate populations in China suggests strong adaptability of the species. Therefore, increased surveillance is warranted to curtail its spread into new areas. Due to limited infestation and potentially disastrous consequences, it may be prudent to consider eradication of *V. hederifolia* from both Nanjing and Zhoushan areas.

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