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REGULAR ARTICLE

Phenotypic plasticity accounts for most of the variation in leaf manganese concentrations in *Phytolacca americana* growing in manganese-contaminated environments

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Abstract
Background and aims *Phytolacca americana*, a globally invasive species, is able to flourish in heavy metal-contaminated habitats. To improve understanding of the adaptive evolutionary mechanisms of plants under heavy metal stress, we investigated key factors contributing to variation in leaf manganese (Mn) content in *P. americana*.
Methods Genetic surveys and common garden experiments were conducted simultaneously in an analysis of *P. americana* populations growing on Mn-contaminated and uncontaminated soil.
Results Our field survey detected a significant relationship between leaf Mn concentrations in *P. americana* and concentrations in the soils from which plants were collected. Microsatellite analyses identified low levels of genetic diversity within and between populations; 32 of 39 populations (82 %) were genetically monomorphic. No genetic differentiation was detected between populations from contaminated and uncontaminated soils. Our common garden experiments showed that Mn concentrations in *P. americana* were related only to the growth habitat, regardless of the origin of the seeds.
Conclusions Combining the results of our ecological and genetic analyses, we concluded that genetic variation is not likely to be responsible for the wide ecological distribution of *P. americana* in China. Rather, phenotypic plasticity is probably the major contributor to its successful colonisation of stressful habitats, such as heavy metal-contaminated soils.

Keywords Phenotypic plasticity · Genetic variation · Hyperaccumulator species · Manganese accumulation · Pokeweed

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Introduction

Heavy metal contamination of soil has received considerable public attention over recent decades. Contamination may be naturally caused or results from anthropogenic activities, such as mining, agriculture, industrial processes and traffic (Baker 1987; Muller et al. 2004). In comparison with plants found at uncontaminated sites, those growing on heavy metal-contaminated soils frequently accumulate elevated

levels of the contaminating metals in their tissues (Antonovics et al. 1971; Brooks et al. 1992; Reeves 2003; Pollard et al. 2014). Some species that evolved on uncontaminated soils have developed tolerant genotypes, which have persisted in toxic environments and expanded their distributions to soils with high concentrations of heavy metals (Kruckeberg 1967). Heavy metal contamination in soil acts as a selective pressure to promote such evolutionary differentiation (Antonovics et al. 1971; Reznick and Ghalambor 2001).

Plants able to accumulate extraordinarily high concentrations of heavy metals in their aerial organs and grow well on metalliferous soils are categorised as hyperaccumulators (Baker and Brooks 1989; Brooks 1998; Krämer 2010; Pollard et al. 2014). The threshold concentration for leaf hyperaccumulation is 2–3 orders of magnitude higher than those in the leaves of most species growing on normal soils and at least one order of magnitude greater than the usual range found in plants from metalliferous soils. Currently, more than 500 plant species are recognised as hyperaccumulators of heavy metals (zinc [Zn], nickel [Ni], manganese [Mn], cobalt [Co], copper [Cu], cadmium [Cd]), metalloids (arsenic [As]) and nonmetals (selenium [Se]) (Verbruggen et al. 2009; van der Ent et al. 2013). These hyperaccumulators may be useful for the removal of toxic metals from contaminated soils; after accumulation in live tissues, the toxins can be removed from the habitat by harvesting metal-rich aboveground plant parts (Baker et al. 2000; Pollard et al. 2002; McGrath et al. 2002; Pilon-Smits 2005; van der Ent et al. 2013). The physiological, molecular and genetic bases of hyperaccumulation have been examined in previous works (Pollard et al. 2002; Macnair 2003; Verbruggen et al. 2009; Krämer 2010). However, the adaptive evolutionary mechanisms of hyperaccumulation under heavy metal stress remain unclear (Pollard et al. 2002; Verbruggen et al. 2009; Cappa and Pilon-Smits 2014). The capacity to tolerate and accumulate metals varies greatly between populations of the same hyperaccumulator species (Pollard et al. 2002; Verbruggen et al. 2009). Studies on phenotypic and genetic variation within/among hyperaccumulator populations will contribute to improved understanding of the evolutionary mechanism(s) underlying heavy metal tolerance (Pollard et al. 2002; Wójcik et al. 2013). The variability of hyperaccumulator populations growing separately in habitats with normal and high concentrations of heavy metals makes these species good models for studying evolution by natural selection.

Genetic differentiation and phenotypic plasticity are two mechanisms by which plants adapt to variable environments (Williams et al. 2008). The genetic variation mechanism operates in species with high genetic diversity; these are able to adapt to local habitats by rapid genetic differentiation; i.e. they are able to evolve (Ward et al. 2008; Bossdorf et al. 2010). The phenotypic plasticity mechanism operates in species whose members are able to shift their phenotypes to adapt to local circumstances without any corresponding genotypic shifts (Bossdorf et al. 2008; Richards et al. 2012). Rapid genetic evolution may occur in only a few generations under the high selection pressure exerted by extreme environmental conditions (Ernst 2006), and the ability of plants to tolerate heavy metals can evolve over relatively short time periods (Bone and Farres 2001). Toxin tolerance and hyperaccumulation ability are at least in part under independent genetic controls (Macnair et al. 1999; Macnair 2002; Assunção et al. 2003) and may vary significantly among and within populations (Escarré et al. 2000; Dechamps et al. 2005; Meyer et al. 2010). Heavy metal-contaminated sites may be considered as ecological islands in which strong selection pressure results in the genetic differentiation of different ecotypes (Lefèbvre and Vernet 1990; Pollard et al. 2002; Muller et al. 2004). A range of studies has identified within-species molecular differences between populations of metal accumulating plants, such as *Silene paradoxa* (Mengoni et al. 2000), *Sedum alfredii* (Deng et al. 2007) and *Viola riviniana* (Kuta et al. 2014). In the field, individual plants of a hyperaccumulating species are often very phenotypically variable, even within a single population (Bert et al. 2002). Phenotypic plasticity reportedly allows plants with the same genotype to cope effectively with environmental heterogeneity (Williams et al. 2008; Richards et al. 2012) and may also contribute to the development of metal tolerance (Bert et al. 2002; Macnair 2002). Possible examples include *Arabidopsis halleri* (Macnair 2002) and *Thlaspi caerulescens* (Baker et al. 1994; Jiménez-Ambriz et al. 2007).

Neutral molecular markers, such as microsatellites, are likely to be useful in identifying metal-accumulative population differences within a species (Mengoni et al. 2001; Pauwels et al. 2005; Jiménez-Ambriz et al. 2007). Quantitative trait analyses through ecological experiments are also likely to provide useful information on the selective forces promoting genetic differentiation, which cannot be obtained by neutral marker analysis

(McKay et al. 2001; McKay and Latta 2002; Bekessy et al. 2003). Therefore, neutral molecular markers and ecological experiments have been combined to study adaptive genetic differentiation among populations (McKay and Latta 2002; Conner and Hartl 2004; Geng et al. 2007).

Phytolacca americana is reportedly a Mn/Cd hyperaccumulator (Tie et al. 2005; Peng et al. 2006, 2008; Gao et al. 2013). The species is a native perennial herb in North America but has become a common invasive alien on metal-contaminated and normal soils in China (Armesto et al. 1983; Pollard et al. 2009). Because of its known history in China, *P. americana* may be used as a model species for studying adaptive evolution under heavy metal stress. In this study, we determined whether the variability in leaf manganese content in *P. americana* results from genetic variation or phenotypic plasticity. We used a combination of ecological experiments and genetic surveys to study adaptive genetic differentiation among populations.

Materials and methods

Sample collection

P. americana was sampled from heavy metal-contaminated and uncontaminated sites. We selected 42 sites (41 sites in six Chinese provinces and 1 site in Japan) in which to collect *P. americana* leaves and soil samples (Table 1). Approximately 30 individuals were collected at each sampling site; the distances between neighbouring plants exceeded 10 m. Portions of the leaf samples were dried with silica gel in preparation for the molecular survey. The remaining leaf samples were washed carefully in tap water, rinsed in deionised water, blotted with tissue paper and dried at 80 °C to constant weight; dry weights were recorded for the chemical analysis. Soil located near the roots of each sampled individual of *P. americana* was also collected for chemical analyses. Soil samples were collected in the 0–20 cm depth range below the surface.

Common garden experiments

Common garden experiments were set up to study Mn uptake by different populations of *P. americana*. All seeds of *P. americana* were grown in a greenhouse operated by Nanjing Agricultural University (average temperature 30/25 °C [day/night]; relative humidity 60–80 %; photoperiod 14/10 h [day/night]). Seeds of *P. americana* were collected from five sites; of these, two were contaminated (TG, TS), two were uncontaminated (MX, JT) (Table 1) and the fifth, an uncontaminated plot (YS) in Yongshun county, was used as the baseline site for the Xiangxi region (total soluble manganese [TSMn]=495 mg kg^{-1}; Peng et al. 2008). Polluted habitats were partitioned by their soil Mn concentrations. The unpolluted habitats were defined as the soil with Mn concentrations lower than 1800 mg kg^{-1} in our field surveys and common garden experiments, which is in accordance with the standards issued by the Ministry of Natural Resources and Environment, Thailand (PCD 1995). We used the Thai soil quality standard for Mn because no equivalent standard exists in China. Healthy, uniform seeds of *P. americana* were selected and germinated in a mixture of perlite and vermiculite held in plastic plates and soaked with tap water for 15 days. After germination, the seedlings were transplanted to plastic buckets containing 2.5 L of half-strength Hoagland nutrient solution (Hoagland nutrient solution: 1 mmol L^{-1} KNO_3, 5 mmol L^{-1} $Ca(NO_3)\cdot 4H_2O$, 5 mmol L^{-1} KH_2PO_4, 2 mmol L^{-1} $MgSO_4\cdot 7H_2O$, 46.26 μmol L^{-1} H_3BO_3, 9.15 μmol L^{-1} $ZnSO_4\cdot 7H_2O$, 0.77 μmol L^{-1} $MnCl_2\cdot 4H_2O$, 0.32 μmol L^{-1} $H_3MoO_4\cdot H_2O$, 0.12 μmol L^{-1} $CuSO_4\cdot 5H_2O$, 20.01 μmol L^{-1} $FeSO_4\cdot 7H_2O$, 20.03 μmol L^{-1} EDTA-2Na·$2H_2O$) and cultivated for 12 days. The seedlings were subsequently treated with three concentrations of Mn (as $MnCl_2$): 9.1 μM (control), 2000 μM and 10,000 μM. Each treatment was replicated in three different plastic buckets, and in each plastic bucket, we planted five seedlings of *P. americana*. We renewed the Hoagland nutrient solution every other day. After 20 days of treatment with Mn, the seedlings were harvested. The lengths of the roots were measured, after which shoots and roots were separated and washed thoroughly with tap water, rinsed in deionised water, blotted with tissue paper and dried at 80 °C to constant weight; dry weights were recorded.

Chemical analyses of soils and plants

Dried plant materials were ground in an agate mortar and digested with concentrated HNO_3 and $HClO_4$ (87:13, *v*/*v*). Soil materials were air-dried at room temperature and further dried at 105 °C for 6 h before subjection to digestion. A mixture of concentrated

Table 1 Details of the *Phytolacca americana* materials used in the studies and concentrations of manganese (Mn) and bioaccumulation factors in sampled sites

Pop. code	Province	Location	Habitat	Latitude	Longitude	TSMn (mg kg^{-1})	LMn (mg kg^{-1})	ESMn (mg kg^{-1})	BAF
FH	Anhui, China	Tongling	Road side	30.9	118.0	2112 (580–5912)	298 (51–1406)	0.3	0.14
SZ		Tongling	Smeltery	30.9	117.9	1143 (303–4245)	4266 (85–14,660)	17.3	3.73
TG		Tongling	Open forest	30.9	117.8	3852 (251–16,710)	1812 (44–4989)	73.1	0.47
BY	Guangxi, China	Laibin	Manganese mine	24.0	109.3	10,432 (3228–50,870)	8349 (2699–15,369)	149.0	0.8
GL		Guilin	Botanic garden	25.1	110.3	546 (62–2748)	917 (36–3705)	18.2	1.68
GP		Guiping	Abandoned mine	23.5	110.3	177,976 (5233–525,684)	7912 (1177–15,128)	72.5	0.04
LP		Lipu	Abandoned mine	24.6	110.4	4212 (268–15,090)	3970 (174–8673)	55.1	0.94
PL		Pingle	Manganese mine	24.7	110.8	14,828 (1312–49,751)	4741 (857–14,698)	105.0	0.32
QZ		Quanzhou	Manganese mine	25.7	111.1	3317 (709–13,881)	2031 (122–5227)	86.7	0.61
TD		Chongzuo	Manganese mine	23.3	107.2	104,503 (8069–283,281)	5902 (608–10,185)	56.1	0.06
XL		Chongzuo	Manganese mine	22.9	106.7	71,630 (11,330–171,590)	14,925 (1364–31,766)	266.0	0.21
CS	Hunan, China	Changsha	Vegetable garden	28.2	113.0	695 (100–1529)	572 (93–4350)	11.4	0.82
CZ		Binzhou	Hill country	25.8	113.0	794 (132–1953)	2302 (42–12,845)	19.4	2.90
HY		Hengyang	Smeltery	26.6	112.6	9888 (814–60,097)	775 (178–3198)	58.8	0.08
JS		Jishou	Smeltery	28.3	109.8	17,771 (110–66,138)	1455 (273–8164)	30.5	0.08
LD		Lengshuijiang	Road side	27.8	111.5	721 (57–2886)	253 (65–1173)	1.2	0.35
XT		Xiangtan	Smeltery	28.0	112.8	29,692 (156–165,470)	2313 (405–5779)	16.4	0.08
YZ		Yongzhou	Smeltery	26.1	111.3	32,011 (276–194,984)	3606 (557–9437)	128.0	0.11
ZJ		Zhangjiajie	Orchard	29.1	110.5	659 (301–942)	114 (62–271)	0.5	0.17
ZX		Xiangxi	Smeltery	28.6	109.5	26,738 (682–102,114)	2506 (130–8379)	1.1	0.09
ZZ		Zhuzhou	Smeltery	27.9	113.1	1131 (178–6267)	346 (59–1485)	0.7	0.31
DQ	Jiangxi, China	Shangrao	Lead zinc mine	29.0	117.6	1958 (37–11,588)	1549 (93–6939)	66.1	0.79
DT		Shangrao	Copper mine	29.0	117.7	480 (99–921)	563 (72–1964)	9.1	1.17
GW		Ganzhou	Gold mine	24.7	114.9	898 (162–4178)	1281 (215–2637)	43.3	1.43
GY		Guixi	Smeltery	28.3	117.2	136 (38–652)	501 (91–2237)	9.7	3.68
HJ		Ganzhou	Farmland	27.0	115.3	136 (16–364)	1150 (167–4162)	27.5	8.43
JK		Shangrao	Gold mine	29.0	117.7	955 (126–2030)	1475 (76–8364)	5.7	1.54
LX		Ganzhou	Village	25.4	114.3	452 (154–599)	810 (50–3906)	42.3	1.79
PW		Ganzhou	Tungsten mine	25.5	114.4	627 (272–906)	633 (176–1640)	5.4	1.01
XW		Ganzhou	Tungsten mine	25.4	114.3	530 (84–1310)	1320 (65–4133)	28.8	2.32
YW		Ganzhou	Tungsten mine	25.7	115.4	2368 (133–23,622)	1520 (47–7666)	3.7	0.64
ZM		Pingle	Farmland	28.8	117.3	22,289 (122–83,848)	2935 (282–7178)	20.6	0.13

Table 1 (continued)

Pop. code	Province	Location	Habitat	Latitude	Longitude	TSMn (mg kg^{-1})	LMn (mg kg^{-1})	ESMn (mg kg^{-1})	BAF
MX	Jiangsu, China	Nanjing	Open forest	32.1	118.8	534 (168–813)	142 (53–414)	10.7	0.27
TS		Nanjing	Copper mine	32.1	119.1	1719 (273–3640)	345 (108–639)	0.4	0.20
QW	Zhejiang, China	Zhuji	Lead zinc mine	29.5	120.3	1352 (550–2156)	441 (62–3883)	86.8	0.33
SX		Shaoxing	Copper mine	29.9	120.6	925 (98–3396)	2023 (27–8831)	13.5	2.19
SY		Shangyu	River side	30.0	120.9	299 (57–2349)	5546 (421–11,584)	37.8	18.55
HZ		Hangzhou	Road side	30.2	120.1	410 (149–656)	894 (61–4237)	5.7	2.18
JH		Jinhua	Road side	29.0	119.7	395 (74–1089)	320 (30–1642)	6.8	0.81
JY		Jinhua	Road side	29.1	119.7	332 (75–1019)	590 (81–4481)	9.7	1.78
JL		Jinhua	Wasteyard	29.1	119.7	483 (49–2353)	1323 (59–4041)	6.0	2.74
JT	Tokyo, Japan	Nishitokyo	Open forest	35.7	139.5	1142 (847–1378)	304 (39–839)	9.7	0.27

Sample number samples for Mn concentration analysis, *TSMn* the total Mn concentration in soil, *LMn* the Mn concentration in leaves, *ESMn* the $CaCl_2$-extractable Mn concentration in soil, *BAF* the bioaccumulation factor

$HF:HNO_3:HClO_4$ (4:1:1, *v*/*v*/*v*) was used to digest the soil materials. The total Mn concentrations in the solutions were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES, Optima 3300 DV, Perkin-Elmer, Waltham, MA, USA).

Five samples were selected randomly from each population for the determination of $CaCl_2$-extractable Mn concentrations in the soils. Air-dried soil materials were mixed with 10 mM $CaCl_2$ for 1 h and then centrifuged (1157×*g*, 15 min). The supernatant solution was mixed with HNO_3 (10:1, *v*/*v*) after filtration through paper and subsequently analysed for $CaCl_2$-extractable Mn by ICP-AES (Optima 3300 DV, Perkin-Elmer).

To determine the concentrations of chlorophyll and carotenoid pigments, the photosynthetic pigments were first extracted by macerating 0.10 g of leaves in a mortar and pestle containing 10 mL 95 % ethanol. The absorptions of the extracts at 470, 649 and 665 nm were measured spectrophotometrically (UV-2450, Shimadzu, Tokyo, Japan). The concentrations (mg g^{-1} fresh leaf mass) of chlorophyll *a*, chlorophyll *b* and total carotenoids were then calculated using the equations of Lichtenthaler and Wellburn (1983).

An ANOVA test was used to distinguish the effects of habitat type (H) and populations (P) on Mn accumulation in leaves. Two habitat group levels were identified using the Thai soil quality standards for habitat and agriculture. SPSS software (ver. 17.0; SPSS Inc., Chicago, IL, USA) was used for the ANOVA. Data were analysed for correlations, and significant differences among means were determined using the LSD test at $P<0.05$.

Molecular surveys

Development of microsatellite markers

We isolated microsatellite regions from *P. americana* using the compound microsatellite marker technique (Lian et al. 2006). In total, 168 sequences were found to contain $(AC)_6$ $(AG)_n$ or $(TC)_6$ $(AC)_n$ compound simple sequence repeat (SSR) motifs, of which 90 possessed unique sequences with sufficient flanking regions for designing specific primers. A specific primer (IP1) was designed from the sequence flanking the compound SSR, and primer pairs, including a specific primer (IP1) and the corresponding compound SSR primer $(AC)_7(AG)_3$ or $(TC)_7(AC)_3$, were used as pairs of compound SSR markers. To examine polymorphisms

with these designed primer pairs, one or two individuals of *P. americana* were chosen randomly from the 39 populations sampled. PCR amplification was performed with Biotaq DNA polymerase (Bioline Ltd., London, UK) in a 5 μL reaction mixture containing ~30 ng template DNA, 2 mM dNTP, 1× NH_4 reaction buffer, 1.35 mM $MgCl_2$, 0.2 U of Biotaq, 0.5 μM of IP1 and 0.5 μM of Texas Red-labelled compound SSR primer, i.e. $(AC)_7(AG)_3$ or $(TC)_7(AC)_3$. The PCR cycling conditions were as follows: 1 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at the annealing temperature for each designed specific primer, 1 min at 72 °C and a 5 min extension at 72 °C for the final cycle. The reaction products were electrophoresed on a 6 % Long Ranger sequencing gel (FMC BioProducts, Rockland, ME, USA) using an SQ-5500E sequencer (Hitachi, Tokyo, Japan). The electrophoretic patterns were analysed using Fraglys software (ver. 3; Hitachi, Tokyo, Japan). Ultimately, only five polymorphic SSR loci were found, and these were used for molecular variation analyses of the 39 populations (Table S1).

Molecular variation analysis

nSSR genotyping We investigated molecular variation within and among the 39 populations using five pairs of SSR loci (Table S1). About 30 individual plants were selected randomly from each population, and 821 of these were genotyped. The PCR analyses were performed as described above.

Genetic diversity To characterise the microsatellite loci in *P. americana*, we used Cervus software (ver. 3.0; Kalinowski et al. 2007) to calculate the following parameters for each locus: the number of alleles (*Na*), observed heterozygosity (*Ho*) and expected heterozygosity (*He*). The number of observed alleles per population (N_A), observed (*Ho*) and expected (*He*) heterozygosities were calculated with GenAlEx software (ver. 6.5; Peakall and Smouse 2012).

Population genetic structure A hierarchical analysis of molecular variance (AMOVA) performed with ARLEQUIN software (ver. 3.5; Excoffier and Lischer 2010) was used to partition the molecular variance within and among populations and among different groups of populations. Fixation indices were computed and tested by permutations for each hierarchical level in the genetic structure: F_{IS} for variation among individuals within populations, F_{SC} for variation among populations within groups and F_{CT} for variation between groups of populations. The significance of each variance component was tested by iterations through 10,000 permutations. To characterise population structure, we performed Bayesian clustering (implemented in Structure ver. 2.3 software; Pritchard et al. 2010) on the entire nSSR dataset through 100,000 Markov chain Monte Carlo (MCMC) cycles (after discarding the first 10,000 cycles as burn-in); we used the admixture model with independent allele frequencies. Ten replications were performed for each K (in the range: K=1–10), and the optimal K was estimated (following Evanno et al. (2005)) based on the parameter ΔK, which evaluates the second-order rate of change of the likelihood function with respect to K.

Results

Manganese accumulation in populations of *P. americana* from contaminated and uncontaminated sites

We found considerable variation in leaf and soil Mn among the 42 sites (Table 1). The maximum and minimum Mn levels in the soils were 178,000 mg kg^{-1} (at site GP) and 136 mg kg^{-1} (at site ZJ), respectively. Although the Mn concentrations in the soils at some sites were high, no Mn toxicity symptoms were seen in *P. americana*. The Mn concentrations in the leaves of different populations also varied greatly between a maximum of 14,900 mg kg^{-1} in the XL population and a minimum of 114 mg kg^{-1} in the ZJ population. On a logarithmic scale, the concentration of Mn in the leaves was significantly correlated with the total concentration of Mn in the soils (Fig. 1, R^2=0.3907, P<0.0001). The relationship between Mn concentration in the leaves and $CaCl_2$-extractable Mn in the soils was also significant (R^2=0.6590, P<0.0001).

The bioaccumulation factor (BAF) is the ratio of metal concentration in the dry plant shoot to that in the soil in which the plant grows. *P. americana* had high BAF ratios. The mean Mn BAF for the 42 sites was 1.58, and BAF values in 38 % of the populations were >1.0 (Table 1). The BAF ratio decreased significantly with increasing Mn concentration in the soils (Fig. 1, R^2=0.6154, P<0.0001).

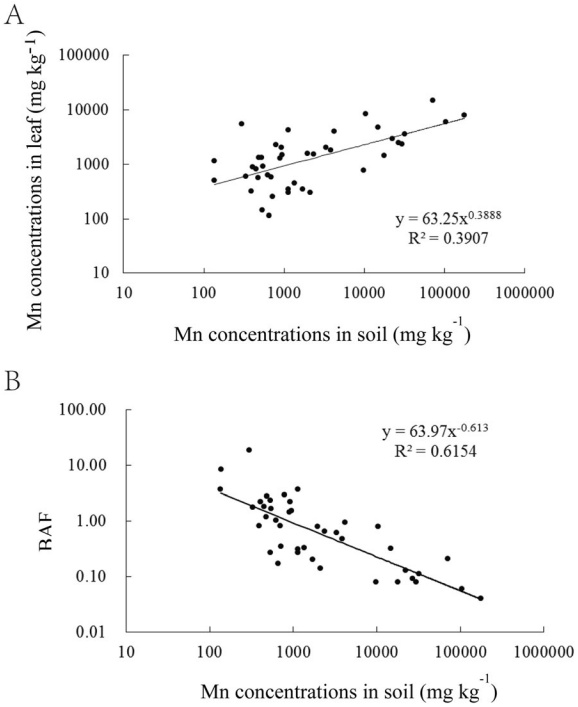

Fig. 1 Relationships between **a** the concentration (mg kg^{-1}) of manganese (Mn) in leaves of *Phytolacca americana* plants and the soil in which they grow and **b** the bioaccumulation factor (BAF) of *P. americana* plants and the concentration (mg kg^{-1}) of Mn in the soil in which they grow

We found a significant effect of Mn habitat type (H) on the Mn concentration in leaves collected by field survey (ANOVA). In general, plants from high-Mn habitats had significantly higher leaf Mn concentrations than those from 'normal' habitats (F=195.00; P<0.01). We also found a significant effect of populations (P) on the Mn concentration in the leaves (F= 50.70; P<0.01).

Plant growth and Mn accumulation in common garden experiments

The Mn concentrations in leaves, stems and roots of all five populations increased markedly with increasing soil Mn concentration (Fig. 2a). At all levels of Mn treatment, the leaves from all five populations accumulated more Mn than the stems or roots. The highest Mn concentration in the leaves of *P. americana* (29,985 mg kg^{-1} dry wt.) occurred in plants from the JT population subjected to the 10 mM Mn treatment. We detected no significant differences in the Mn concentrations of roots, stems or leaves among the five populations. In contrast, the effects of Mn treatments on growth were not consistent among the five populations (Fig 2b, c). The 2 mM Mn treatment inhibited root elongation in YS but had an enhancing effect on root growth in the remaining four populations. The biomass of the roots and shoots subjected to this treatment increased in the YS and TS populations but decreased in the other three. Root elongation was reduced in the YS and JT populations but enhanced in the other three populations subjected to 10 mM Mn treatment. The biomasses of roots decreased in all of five populations subjected to this treatment; shoot biomasses increased in the MX and JT populations but decreased in the other three populations. The concentrations of chlorophyll *a*, chlorophyll *b* and carotenoid pigments of all five populations decreased with increasing Mn concentrations in the nutrient solutions (Fig 2d).

Molecular variation analysis

In total, we assessed 821 individuals from 39 populations of *P. americana* by microsatellite analysis. We detected a total of 17 alleles in the survey of five microsatellite loci; 2–4 alleles were detected for each locus (Table S1) and 16 genotypes identified (Table S2). The expected (*He*) heterozygosity of the loci estimated across all populations ranged from 0.045 to 0.094. Among the 39 populations, 32 (82 %) were monomorphic for one dominant genotype (A). The remaining seven populations had more than one genotype (Table S3). The observed heterozygosity was zero in all populations; thus, all alleles within the individuals were homozygous. Across all populations, population MX had the largest number of alleles per locus (2.8) and the highest expected heterozygosity (0.53).

The AMOVA tested whether population groupings based on geographical distributions or on habitat pollution were related to an uneven distribution in genetic variance (Table 2). To group the 39 populations geographically, we divided them into seven groups by subordinate provinces and counties. We classified populations into two habitat pollution groups: polluted and unpolluted, based on the Mn concentrations in the soils. We found no significant genetic variance attributable to grouping based on geographic location (P=0.061) or habitat pollution (P=0.828). A hierarchical AMOVA demonstrated that only a small proportion of genetic variance (0.35 %) was explained by grouping into polluted and unpolluted habitats, compared to 44.65 % of

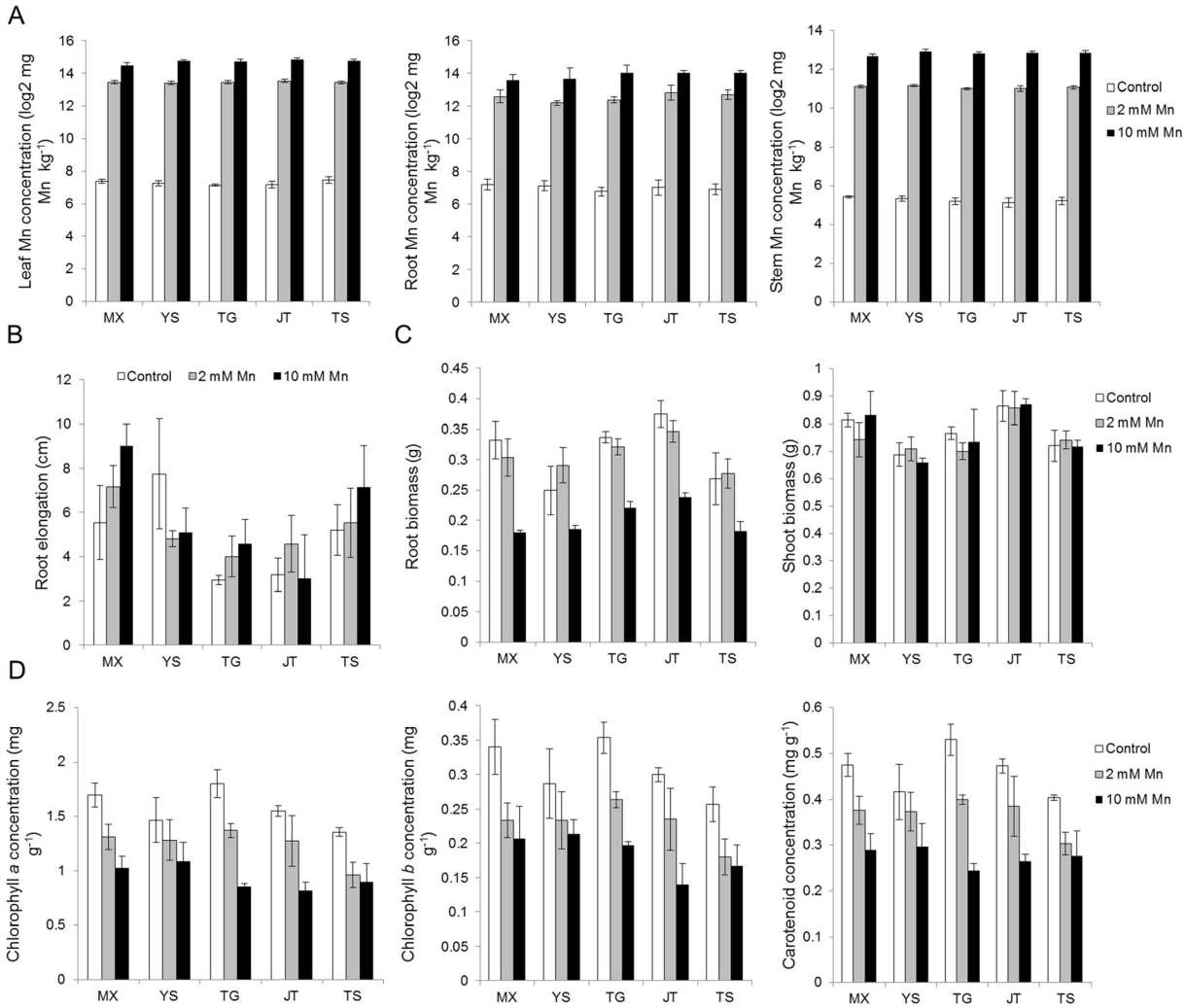

Fig. 2 **a** The concentration (mg kg^{-1}) of manganese (Mn) in the different parts of *Phytolacca americana* including leaves, stems and roots. **b** Root elongation. **c** The biomass of roots and shoots. **d** The concentrations (mg g^{-1}) of chlorophyll *a*, chlorophyll *b* and carotenoids in control, 2 mM and 10 mM Mn treatments over 20 days

variance among populations within these habitat groups ($P<0.001$) and 55.00 % within populations ($P<0.001$). We estimated a moderate proportion genetic variance (21.25 %) attributable to grouping into seven geographical categories.

In our structure analysis, the number of clusters (K) in the data should have been four since the ΔK statistic of Evanno et al. (2005) allows detection of a rate change in ln p(D) corresponding to $K=4$ (Fig. 3a). All individuals were assigned to four clusters (clusters 1 [red], 2 [green], 3 [blue] and 4 [yellow]) (Fig. 3b). Most individuals (93.7 %) from the 42 sites fell within clusters 1 (28 %), 2 (36 %) and 3 (36 %). Some individuals from the MX and GL populations were assigned to cluster 4, and they accounted for 51.3 and 15 % of individuals in these two populations, respectively. Most individuals in the QZ population (96.4 %) were assigned to cluster 1. When combined with the results of the AMOVA, this procedure demonstrated that there was no correlation between the clusters by structure analysis or by pollution habitat.

Discussion

We used a combination of ecological experiments and genetic analyses to study the major factors influencing heavy metal variation in the leaves of *P. americana*. We found no genetic variation between populations of

Table 2 Hierarchical analysis of molecular variance for microsatellite variation surveyed in the 39 populations of *Phytolacca americana*

Source of variation	d.f.	Sum of squares	Variance	% total	*F*, *P* value
Geographical partition[a]					
Between groups	6	61.13	0.0365	21.25	F_{CT}=0.212 *P*=0.061
Among populations within groups	32	64.33	0.04424	25.76	F_{SC}=0.327***
Within populations	782	142.353	0.09102	52.99	F_{IS}=1.00***
Total	820	267.81	0.17176		
Polluted habitats partition[b]					
Between groups	1	3.75	0.00057	0.35	F_{CT}=0.003 *P*=0.828
Among populations within groups	7	121.71	0.07389	44.65	F_{SC}=0.448***
Within populations	782	142.35	0.09102	55.00	F_{IS}=1.000***
Total	820	267.81	0.16549		

d.f. degrees of freedom

***$P<0.001$

[a] Geographical partition according to population subordinate provinces and countries

[b] Polluted habitats partition according to the Mn concentration in soil, and the maximum soil Mn for unpolluted habitat was 1800 mg kg^{-1} based on soil quality standards for habitat and agriculture in Thailand

P. americana from contaminated and uncontaminated sites. Leaf Mn concentrations were related to habitat conditions and not to the provenance of the seeds.

Our field surveys showed that *P. americana* is highly adapted to a wide range of soil Mn concentrations. In the XL population, leaf Mn concentrations reached 14,900 mg kg^{-1}. A comparison with the range of foliar Mn levels across diverse plant species (Reeves and Baker 2000; Epstein and Bloom 2005; Marschner 2011) leaves no doubt that *P. americana* is highly tolerant of heavy metal contamination. The species is also highly tolerant of cadmium pollution (Liu et al. 2010).

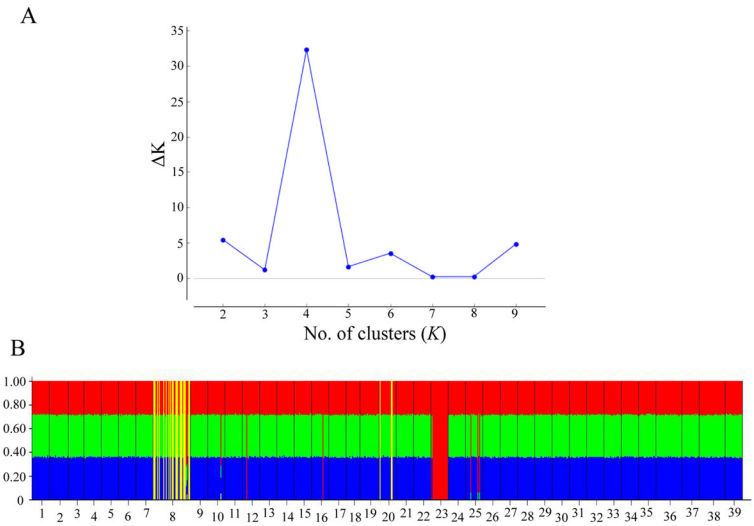

Fig. 3 Summary of structure analyses based on nuclear microsatellite (nSSR) data collected from 821 individuals (in 39 populations) of *Phytolacca americana*. *Numbers* of clusters (*K*) varied from *1 to 10* in 10 independent runs. **a** Plot of the corresponding ΔK statistics calculated using the procedures Evanno et al. (2005). **b** *Bar diagram* of a structure analysis for the model with K=4 (i.e. with the highest ΔK). Each *colour* corresponds to a suggested cluster, and each *vertical bar* represents a single individual. The *y*-axis indicates the proportion of an individual's genome assigned to a given cluster. The *x*-axis refers to the population number (e.g. 1 = JK)

Persistence under these adverse conditions may reflect adaptive evolution in *P. americana*.

Our genetic diversity survey aimed to determine whether there was a relationship between genetic variation and *P. americana* adaptation to Mn-contaminated habitats. We designed and tested many primers; however, only five pairs of microsatellite primers were polymorphic. We found little or no genetic diversity among the populations of *P. americana* we studied in China. With the exception of the MX population (H_E=0.53), little genetic diversity was observed in either contaminated or uncontaminated populations. Most populations contained only one widely distributed genotype (A). All 821 individuals in the 39 populations examined were homozygotic, suggesting that selfing may be the main mating system of *P. americana*, as reported in previous studies; clonal reproduction also occurs, but apomixis has not been observed (Armesto et al. 1983).

Outcrossing alien species that have been introduced to new locations on multiple occasions generally have extensive genetic variation from which locally adapted ecotypes differentiate in a wide range of habitats under the influence of natural selection (Bossdorf et al. 2010). In contrast, some invaders with fewer introductions, inbreeding and clonal reproduction usually have low levels of genetic variation. Meanwhile, Dlugosch and Parker (2008) found that a decrease in genetic variance did not reduce the species' phenotypic variance in their study. Based on the structure analysis, we concluded that *P. americana* in China originated from four ancestral gene pools. Three (clusters 1, 2 and 3) had already been mixed in a single pool by hybridisation before the species was introduced to China. Most of the populations in China were members of this mixing pool and had representative genotype A; the MX population came from another ancestral gene pool (cluster 4). Thus, *P. americana* in China may have been introduced on only a few occasions, perhaps just one. According to existing records, native North American *P. americana* was introduced into China less than a century ago (Pollard et al. 2009; Qiang unpublished information). The founding populations in China may contain only subset of the genetic diversity in North American populations. Extant Chinese populations of *P. americana* growing in different habitats may represent genetically identical clonal offspring of a single ancestor.

Baker (1965) was the first to indicate that some colonising species may possess 'general-purpose genotypes'. Bossdorf et al. (2008) further suggested that these species are able to thrive in a wide range of environmental conditions through adaptive phenotypic or developmental plasticity. Phenotypic plasticity can increase fitness and may contribute to plant persistence in a wide range of habitats (Richards et al. 2008, 2012). Genotype A of *P. americana* may also be a general-purpose genotype. In such clonally invasive species, plasticity may emerge as the primary strategy for coping with conditions in a wide range of habitats (Geng et al. 2007). The phenotypic variation among *P. americana* individuals that we encountered in the field may be attributed to phenotypic plasticity, rather than ecotypic differentiation. Thus, even though surprisingly large losses of heterozygosity have occurred in *P. americana*, phenotypic plasticity may contribute to the persistence of this species in adverse environments, such as a heavy metal-contaminated habitats. Moreover, *P. americana* has traits that favour range expansion, including features that promote long distance dispersal of seeds by birds; these traits contribute to overcoming the disadvantage of limited genetic variation (Armesto et al. 1983; Orrock 2005). Thus, *P. americana* has become a successful invader in China and has adapted to a range of heavy metal-contaminated environments.

On their own, the data obtained from microsatellite marker analysis do not discount the possibility of a genetic strategy for evolutionary adaptation to local habitats because microsatellites are neutral molecular markers that may not capture the genetic differentiation of an ecologically important quantitative trait (McKay et al. 2001; Bekessy et al. 2003). Thus, common garden experiments were also necessary to provide a robust test able to differentiate potential plasticity from genetic variation strategies of *P. americana*. In our comparison of Mn uptake by five *P. americana* populations from different habitats grown together in common garden experiments, we found that the Mn concentrations in the roots and shoots of all five populations increased markedly with increasing Mn concentration in the growth medium. No significant differences in plant tissue Mn concentrations were found between the five populations. The Mn concentrations in *P. americana* were related only to the Mn concentrations in the habitat, regardless of the origin of the seeds. Thus, we found no ecotype differentiation across the populations of *P. americana* from different habitats. Pollard et al. (2009) reported that *P. americana* populations in the native range in the southeastern USA also have a latent physiological ability to hyperaccumulate Mn.

Most known Mn hyperaccumulators are woody species (Reeves and Baker 2000). Fernando et al. (2007) studied the natural variability in leaf Mn accumulation in the tree species *Gossia bidwillii*, which is a Mn hyperaccumulator, and demonstrated that Mn hyperaccumulation occurred across multiple populations over a large region regardless of the substrate Mn supply. These data, in combination with a genetic diversity experiment, suggest that genetic variation is unlikely to account for the abundance of *P. americana* across a wide range of habitats in China. Thus, phenotypic plasticity may be the major contributor to the success of *P. americana* in colonising stressful habitats, such as heavy metal-contaminated soils. Similar patterns have been reported in *Thlaspi caerulescens*; Jiménez-Ambriz et al. (2007) found that the divergent selection pressure exerted by soil toxicity played a predominant role in shaping life history differences between ecotypes of *T. caerulescens* in southern France. Gene flow weakly opposed local adaptation, despite the geographical proximity of populations studied.

Several recent investigations have shown that phenotypic plasticity can be mediated through epigenetic effects (Scoville et al. 2011; Kilvitis et al. 2014). Epigenetic states may be altered when organisms are exposed to stressful or novel environments, and these epigenetic shifts can rapidly generate a source of phenotypic variation without any change in genetic variation, thereby ultimately affecting the evolution of populations (Bossdorf et al. 2008; Richards et al. 2012). For a better understanding of adaptive evolution in *P. americana*, future analyses of amplified fragment length polymorphism (AFLP) and methylation sensitive-AFLP (MS-AFLP) will be combined to compare genetic and epigenetic diversity between heavy metal-contaminated and normal habitats.

Conclusions

Mn concentrations in *P. americana* leaves were found to be largely dependent on soil Mn concentration. Under common hydroponic conditions, populations collected from uncontaminated and contaminated sites accumulated similar amounts of Mn in their leaves, suggesting that ecotype differentiation had not occurred among the populations of *P. americana* growing in different habitats. We detected limited genetic variation among populations in China and no genetic differentiation between populations from contaminated and uncontaminated soil. Thus, genetic variation may not account for the success of *P. americana* across a wide range of habitats in China. The plasticity of ecologically relevant traits, such as Mn accumulation, plays a major role in the success of this invasive species in colonising heavy metal-contaminated environments.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no competing interests.

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