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Associations between population epigenetic differentiation and environmental factors in the exotic weed mile-a-minute (*Mikania micrantha*)

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Research Article

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Abstract

Invasive species face new selective pressures and low genetic variation caused by genetic bottlenecks and founder effects when they are introduced into novel environments. Epigenetic variation may help them to cope with these problems. Mile-a-minute (*Mikania micrantha* Kunth) is a highly invasive exotic weed that has seriously damaged biodiversity and agricultural ecosystems. We first adopted methylation-sensitive amplified polymorphism (MSAP) markers to investigate epigenetic variation of 21 *M. micrantha* populations in southern China, and further explored the effects of environmental factors on population epigenetic differentiation by correlating epigenetic and climate and soil data. Adaptive epiloci positively correlated with climate/soil variables were identified. Minimum temperature of the coldest month and mean temperature of the coldest quarter were considered as decisive factors for its distribution. Climate is presumed to play a relatively more important role than soil in shaping the adaptive epigenetic differentiation in *M. micrantha*. Under ongoing global warming, populations of *M. micrantha* are predicted to expand northward. In addition, the weed also presented higher epigenetic variation compared with genetic variation. Leaf shape variation was detected related to methylation-state change at the population level.

Introduction

How species can become invasive is a core issue in invasive biology. Invasive species usually face new selective pressures when they are initially introduced into novel environments. How to deal with these pressures directly determines their fate (Matesanz et al. 2010). Generally, invasive species are expected to have low genetic variation, due to genetic bottlenecks and founder effects (Dlugosch and Parker 2008), which may not be enough to support their survival (Crawford and Whitney 2010). However, they are still able to successfully colonize new habitats and defeat native species. Epigenetic variation may play a critical role in this process and may facilitate invasive species' rapid response to environmental stress (Ni et al. 2018).

Revealing populations' epigenetic variation has attracted increasing attention from invasive biologists. Independent from genetic control, epigenetic modifications can be directly induced by environmental stimuli and are reflected in phenotypic changes through modulation of gene expression without changing the underlying DNA sequences (Ni et al. 2018; Richards et al. 2017; Shi et al. 2019). As an additional, accelerated pathway to evolutionary change (Bossdorf et al. 2008), epigenetic modifications of various types, such as DNA methylation, modifications on histones and other chromosomal proteins, and the generation of extrachromosomal regulatory small RNAs and noncoding RNAs, have been widely described in eukaryotes (Ni et al. 2018; Richards et al. 2017). Among these, DNA methylation is regarded as the most common type of epigenetic variation and has been intensively studied for eukaryotes. DNA methylation is referred to as the addition of a methyl group to cytosine to form 5-methylcytosine. It has been shown to function in multiple biological processes, including hindering transcription initiation, restraining transcription elongation, silencing transposons, inactivating the X chromosome, and shaping phenotypic variation (Akimoto et al. 2007; Jones 2012; Ni et al. 2018). For plants, cytosine methylation is maintained by methyltransferase, chromomethylase, or an RNA-dependent DNA methylation pathway; it tends to position in symmetric (CG, CHG) and asymmetric (CHH) contexts (H = A, C, or T) and mainly distribute in transposons and repeat regions (Henderson and Jacobsen 2007). In non-model plants, the global DNA methylation state can be screened in natural populations using methylation-sensitive amplified polymorphism (MSAP) markers (Angers et al. 2010), a restriction enzyme-based modified amplified fragment-length

Table 1. Sampling information of *Mikania micrantha* populations.

ID	Population[a]	No. of samples	Longitude	Latitude	Altitude
1	DG2	15	113.800°E	22.879°N	48
2	DG3	15	113.768°E	22.905°N	182
3	DG4	15	113.793°E	22.854°N	113
4	HK1	15	114.043°E	22.489°N	4
5	HK3	15	114.234°E	22.391°N	80
6	HK4	15	114.261°E	22.527°N	4
7	HK5	10	114.153°E	22.397°N	227
8	HK6	15	114.197°E	22.257°N	100
9	HK7	11	114.142°E	22.451°N	39
10	HK8	10	114.134°E	22.387°N	48
11	MA1	15	113.556°E	22.140°N	10
12	MA4	16	113.563°E	22.122°N	135
13	NLD2	15	113.817°E	22.409°N	48
14	NLD3	16	113.804°E	22.404°N	11
15	NLD5	15	113.800°E	22.420°N	2
16	NLD6	16	113.811°E	22.415°N	24
17	SZ1	16	114.058°E	22.554°N	47
18	SZ4	15	114.037°E	22.574°N	39
19	SZ5	15	114.170°E	22.580°N	85
20	ZH1	15	113.631°E	22.428°N	6
21	ZH2	16	113.678°E	22.414°N	13

[a]DG, Dongguan; HK, Hong Kong; MA, Macao; NLD, Nei Lingding Island; SZ, Shenzhen; ZH, Zhuhai.

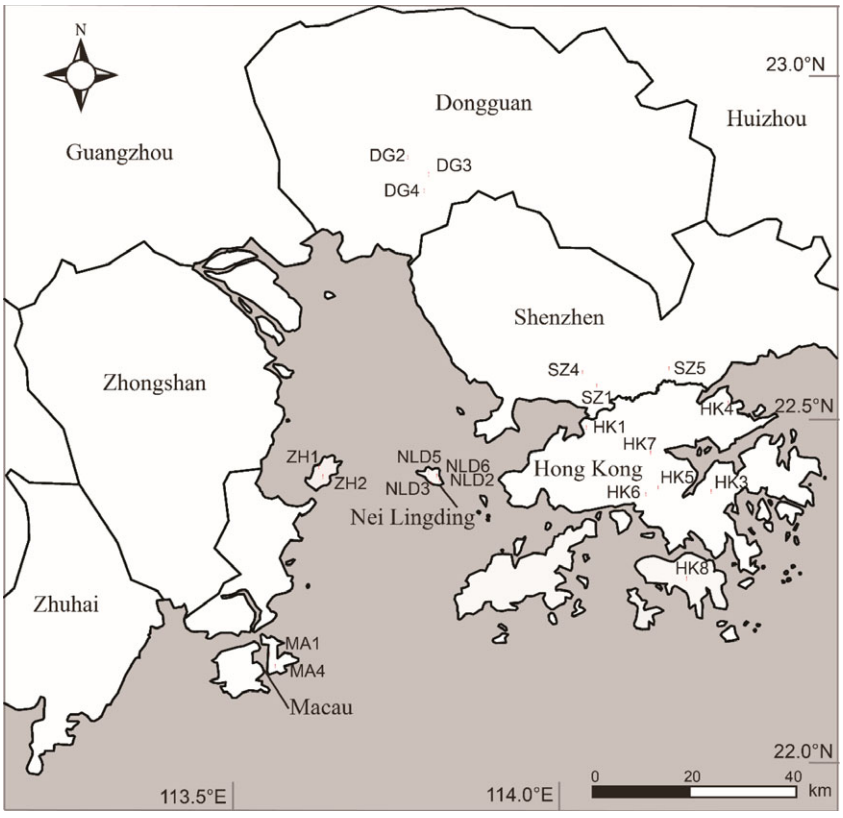

Figure 1. Sampling map of 21 *Mikania micrantha* populations in southern China. See Table 1 for population codes.

polymorphism (AFLP) technique. In addition, the MSAP technique can also provide a quick glance for potential effects of environmental factors on population epigenetic differentiation.

In a heterogeneous environment, climate may exhibit high spatial variation that acts as an important abiotic factor to promote adaptive evolution of plants (Abdala-Roberts and Marquis 2007; Savolainen et al. 2007). Climate not only profoundly influences seed germination, productivity, and distribution of plants, but also controls other critical variables like the length of a wet or dry season (Concilio et al. 2009; Poncet et al. 2010). Correlations between

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Table 2. Information for adopted adapters and primers used in this study.

Primers	Sequences
Adapters	
*Eco*RI–adapter top	5′-CTCGTAGACTGCGTACC-3′
*Eco*RI-adapter bottom	5′-AATTGGTACGCAGTCTAC-3′
*Hpa*II/*Msp*I-adapter top	5′-GATCATGAGTCCTGCT-3′
*Hpa*II/*Msp*I -adapter bottom	5′-CGAGCAGGACTCATGA-3′
Pre-PCR primers	
*Eco*RI + CA	5′-GACTGCGTACCAATTCA-3′
*Hpa*II/*Msp*I	5′-ATCATGAGTCCTGCTCGG-3′
Selective PCR primers	
*Eco*RI + AG (E2)	5′-GACTGCGTACCAATTCAAG-3′
*Eco*RI + CG (E3)	5′-GACTGCGTACCAATTCACG-3′
*Eco*RI + CT (E4)	5′-GACTGCGTACCAATTCACT-3′
*Eco*RI + CC (E5)	5′-GACTGCGTACCAATTCACC-3′
*Eco*RI + GC (E7)	5′-GACTGCGTACCAATTCAGC-3′
*Eco*RI + GG (E8)	5′-GACTGCGTACCAATTCAGG-3′
*Hpa*II/*Msp*I + TAA (H/M1)	5′-ATCATGAGTCCTGCTCGGTAA-3′
*Hpa*II/*Msp*I + TCC (H/M2)	5′-ATCATGAGTCCTGCTCGGTCC-3′
*Hpa*II/*Msp*I + TTC (H/M3)	5′-ATCATGAGTCCTGCTCGGTTC-3′
*Hpa*II/*Msp*I + TAG (H/M6)	5′-ATCATGAGTCCTGCTCGGTAG-3′
*Hpa*II/*Msp*I + TTG (H/M7)	5′-ATCATGAGTCCTGCTCGGTTG-3′
*Hpa*II/*Msp*I + TCA (H/M8)	5′-ATCATGAGTCCTGCTCGGTCA-3′

climatic factors and epi-adaptive evolution have been found in a number of plants. For instance, an adaptive epigenetic memory of the local temperature prevailing during zygotic embryogenesis and seed maturation has been observed in the progeny of Norway spruce [*Picea abies* (L.) Karst.] (Yakovlev et al. 2011). Associations between DNA methylation variation and climate variables were also detected in *Arabidopsis thaliana*: CHH methylation was found to increase with temperature and concentrate in transposable elements, whereas CG methylation showed a correlation with the latitude of origin and primarily occurred on genic regions (Dubin et al. 2015; Keller et al. 2016). These findings underscore the contribution of epigenetic variation to local adaptation. Currently, rapid climatic change is exerting new selection pressure on plants, which will eventually affect their adaptability (Corre and Kremer 2012; Eveno et al. 2008; Jump et al. 2006). For invasive species, investigating the contribution rate of climatic factors to epi-adaptation may provide better understanding of the mechanisms underlying response to climate change and improve the prediction for expansion speed and area.

Soil is another crucial factor associated with plant adaptive evolution (Hancock et al. 2011). It provides essential nutrients for plants, including water, mineral matter, organic matter, and metal elements. Any subtle change in soil metal content has the potential to drive local adaptation of plants (Alberto et al. 2010). Because changes in soil compositions have a strong impact on biochemical and physiological processes of plants, they are frequently posited to be an important driver of divergent selection (Lechowicz and Bell 1991; Macel et al. 2007). Soil factor–driven population-level genetic differentiation and adaptive loci associated with soil properties and metal content have been found in plants such as [*Arabidopsis halleri* (L.) O'Kane & Al-Shehbaz] (Meyer et al. 2009), common gum cistus (*Cistus ladanifer* L.) (Quintela-Sabarís et al. 2012), and [*Eucalyptus tricarpa* (L.A.S. Johnson) L.A.S. Johnson & K.D. Hill] (Steane et al. 2015). It is of note that changes in soil water content, temperature, and organic matter are closely related to climate change, so specific plant genotypes may be generated under the simultaneous selection by climate and soil factors (Fischer and Whitham 2014). As for epipopulation genetics, Kim et al. (2016) have reported the soil metal–associated adaptive methylation variation in red maple (*Acer rubrum* L.).

Mile-a-minute (*Mikania micrantha* Kunth; tribe Eupatorieae, family Asteraceae) is a highly invasive weed. It is a perennial terrestrial herbaceous vine with a slender and branched stem, having a creeping or twining growth habit. As its common name indicates, *M. micrantha* shows a strong invasiveness, which is due to its efficient sexual and vegetative reproduction coupled with wind-dispersed seed (Swamy and Ramakrishnan 1987). It also demonstrates a strong phenotypic plasticity in the introduced environmental conditions (Hong et al. 2006; Wen et al. 2000; Xu et al. 2013, 2014). This weed is highly destructive for local plants, smothering them and blocking sunlight. *Mikania micrantha* has caused serious damage to local biodiversity and agricultural ecosystems. Originating from Latin America, *M. micrantha* was introduced into Hong Kong in 1884 for horticultural purposes (Wang et al. 2003). It became naturalized in 1919 and started to expand on a large scale in southern China since 1984 (Wang et al. 2003). With recent climatic change, *M. micrantha* has shown the potential to invade to inland provinces such as Jiangxi in China (Hu et al. 2014).

In this study, we used MSAP, in junction with environmental conditions, to explore the (epi)genetic variation and local adaptation of introduced *M. micrantha* populations in southern China. Our specific objectives are: (1) to estimate and compare the amount and structuring of genetic and epigenetic variation in the introduced populations; (2) to identify the candidate (epi) loci for selection that are associated with local climatic/soil factors; and (3) to search for the selective effects of environmental variables on population expansion. In addition, we examined the correlation between leaf shape variation and methylation-state change.

Materials and Methods

Sample Collection

To cover as much of the substantial environmental gradient of *M. micrantha* as possible, we collected 306 individuals from 21 invasive populations in Dongguan, Nei Lingding Island, Hong Kong, Macao, Shenzhen, and Zhuhai in southern China (Figure 1; Table 1). Leaves were first stored in silica gel and then at −20 C until DNA extraction. Total genomic DNA was isolated using the modified CTAB method (Porebski et al. 1997). The quality and quantity of DNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). In addition, we measured the leaves of *M. micrantha* in different populations with the same growth period. Length:width ratio was used as a metric of leaf phenotypic variation.

MSAP Assay

We selected a pair of isoschizomers, *Msp*I and *Hpa*II, which recognize and cleave the same 5′-CCGG-3′ sequence with different sensitivities to the methylation at the internal or external cytosine (Schulz et al. 2013). Total genomic DNA was digested at 37 C for 3 h in two parallel reactions using 10 U *Eco*RI and 5 U *Hpa*II or 10 U *Msp*I (New England Biolabs, Beverly, MA, USA) in a final volume of 20 µl, followed by 65 C for 20 min to inactivate the enzymes. *Eco*RI and *Hpa*II/*Msp*I adapters were ligated to digested products, and the reaction was carried out in a 20-µl volume containing 5 pmol adaptor and 60 U of T4 DNA ligase (New England

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Table 3. Methylation status, band pattern, and scoring methods.

Type	Methylation status	Band pattern	Salmon Scoring	AFLP Scoring	Mixing Scoring 2		
					H	M	U
I	CCGG GGCC	11	0	1	0	0	1
II	C^{m}CGG GG^{m}CC C^{m}CGG GGCC	01	1	1	0	1	0
III	^{m}CCGG GGCC	10	1	0	1	0	0
IV	^{m}CCGG GGC^{m}C ^{m}C^{m}CGG GG^{m}C^{m}C ^{m}C^{m}CGG GGCC ^{m}C^{m}CGG GG^{m}CC ^{m}C^{m}CGG GGC^{m}C ^{m}CCGG GG^{m}CC mutation	00	0	0	0	0	0

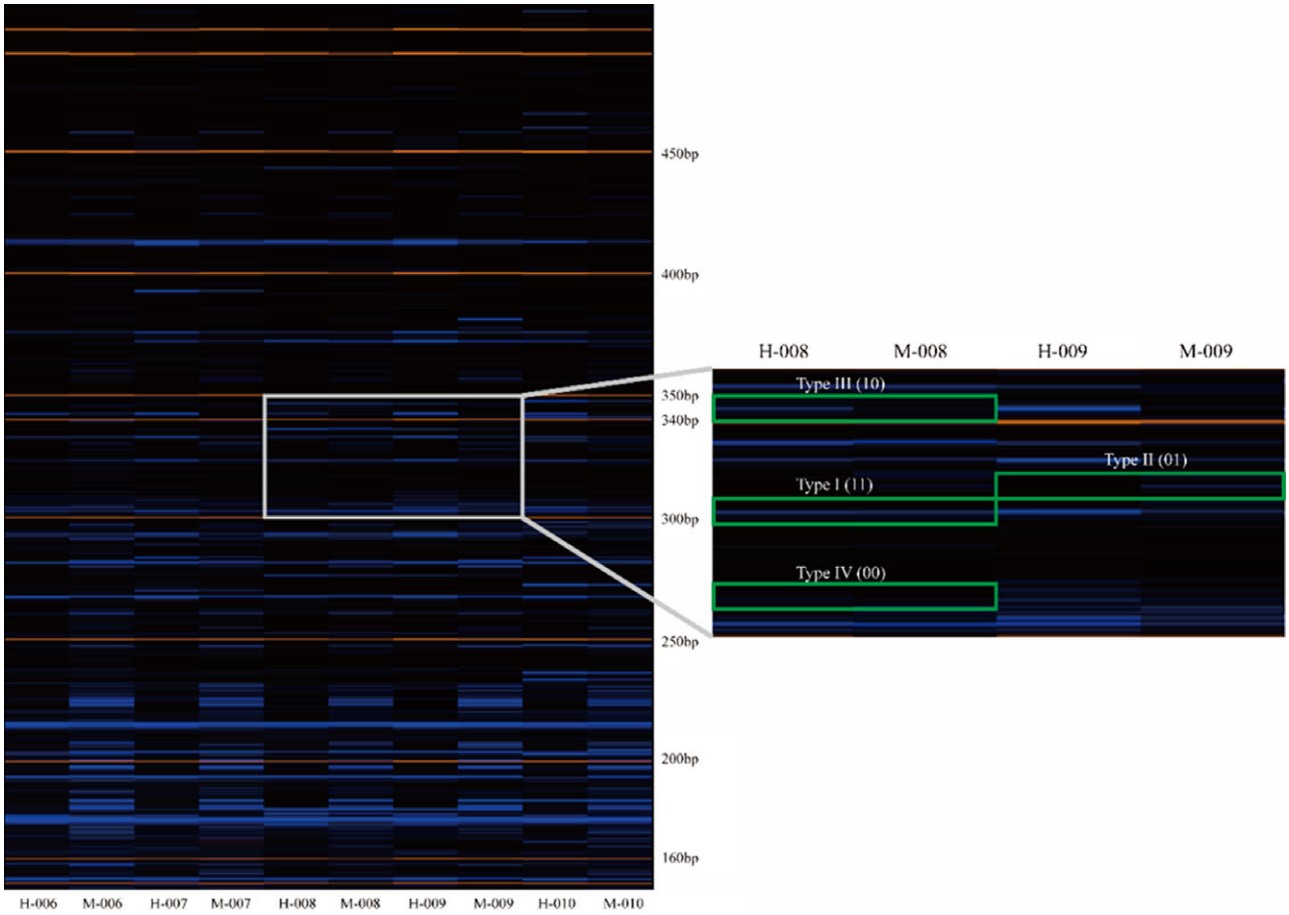

Figure 2. Methylation-sensitive amplified polymorphism (MSAP) electrophoresis was performed on an ABI 3730 DNA analyzer with internal size standard LIZ 500 for E7/H8 primer combination. H and M represent digestion with *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I, respectively. Blue and orange bands show the amplification products and the standard marker, respectively.

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Table 4. Genetic and epigenetic diversity in regions based on the six matrices.

Population[b]	*N*	N_a	N_e	*I*	*%P*	H_e	UH_e
MS-AFLP							
DG	45	1.046	1.135	0.145	52.1%	0.088	0.089
HK	91	1.472	1.102	0.129	73.6%	0.072	0.072
MA	31	0.626	1.057	0.072	31.1%	0.041	0.041
NLD	62	1.164	1.125	0.139	58.0%	0.082	0.083
SZ	46	1.077	1.121	0.134	53.7%	0.080	0.080
ZH	31	0.728	1.104	0.110	35.9%	0.067	0.068
Average	55	1.077	1.108	0.124	53.7%	0.072	0.073
Total	306	2.000	1.116	0.141	100.0%	0.079	0.080
Salmon							
DG	45	1.072	1.131	0.146	53.6%	0.087	0.088
HK	91	1.571	1.117	0.148	78.6%	0.083	0.083
MA	31	1.011	1.103	0.126	50.5%	0.072	0.073
NLD	62	1.249	1.126	0.147	62.5%	0.086	0.086
SZ	46	1.082	1.112	0.132	54.1%	0.077	0.077
ZH	31	0.844	1.106	0.120	41.9%	0.071	0.072
Average	55	1.197	1.118	0.140	59.8%	0.081	0.082
Total	306	2.000	1.119	0.152	100.0%	0.084	0.084
H							
DG	45	0.819	1.065	0.083	41.0%	0.046	0.047
HK	91	1.497	1.077	0.110	74.9%	0.058	0.058
MA	31	0.972	1.086	0.111	48.6%	0.062	0.063
NLD	62	1.074	1.065	0.089	53.7%	0.048	0.048
SZ	46	0.837	1.053	0.075	41.9%	0.040	0.040
ZH	31	0.663	1.059	0.076	33.1%	0.042	0.043
Average	55	1.040	1.069	0.094	52.0%	0.051	0.051
Total	306	2.000	1.068	0.102	100.0%	0.052	0.052
M							
DG	45	1.088	1.119	0.136	54.4%	0.080	0.081
HK	91	1.348	1.077	0.103	67.4%	0.056	0.056
MA	31	0.505	1.042	0.054	25.2%	0.030	0.030
NLD	62	1.185	1.111	0.132	59.2%	0.076	0.076
SZ	46	1.046	1.106	0.124	52.3%	0.072	0.073
ZH	31	0.677	1.088	0.097	33.4%	0.058	0.059
Average	55	1.034	1.091	0.110	51.7%	0.063	0.063
Total	306	2.000	1.094	0.124	100.0%	0.067	0.068
U							
DG	45	0.684	1.065	0.076	34.2%	0.044	0.044
HK	91	1.409	1.058	0.087	70.5%	0.044	0.044
MA	31	0.546	1.037	0.052	27.3%	0.028	0.028
NLD	62	0.922	1.060	0.077	46.1%	0.042	0.043
SZ	46	0.776	1.055	0.069	38.8%	0.038	0.039
ZH	31	0.533	1.054	0.063	26.6%	0.036	0.037
Average	55	0.867	1.055	0.072	43.4%	0.039	0.040
Total	306	2.000	1.057	0.083	100.0%	0.042	0.042
HMU							
DG	45	0.866	1.081	0.097	43.3%	0.056	0.056
HK	91	1.431	1.072	0.102	71.5%	0.054	0.054
MA	31	0.727	1.061	0.079	36.3%	0.044	0.044
NLD	62	1.069	1.078	0.099	53.5%	0.055	0.055
SZ	46	0.884	1.070	0.088	44.2%	0.049	0.050
ZH	31	0.635	1.066	0.079	31.6%	0.046	0.046
Average	55	0.995	1.072	0.093	49.8%	0.051	0.052
Total	306	2.000	1.073	0.104	100.0%	0.054	0.054

[a]*N*, number of samples; N_a, number of different alleles; N_e, number of effective alleles = $1/(p^2+q^2)$; *I*, Shannon's diversity index = $-1*[p*\mathrm{Ln}(p)+q*\mathrm{Ln}(q)]$; *%P*, percentage of polymorphic loci; H_e, expected heterozygosity = $2*p*q$; UH_e, unbiased expected heterozygosity = $[2N/(2N-1)]*H_e$.
[b]DG, Dongguan; HK, Hong Kong; MA, Macao; NLD, Nei Lingding Island; SZ, Shenzhen; ZH, Zhuhai.

Biolabs). After incubation at 16 C overnight, the reaction was heat deactivated at 65 C for 20 min. All adapter and primer sequences for the MSAP protocol are listed in Table 2.

A preselective polymerase chain reaction (PCR) was performed in a total volume of 20 µl, containing 10 µl of the ligation product, 5 pmol of each preselective primers, 1 U *Taq* DNA polymerase (Takara), and 3.75 mmol dNTP (with Mg^{2+}). Thermocycling conditions were as follows: 94 C for 5 min followed by 20 cycles of 94 C for 40 s, 56 C for 45 s, and 72 C for 1 min. After preselective amplification, the PCR products were diluted 1:50 with sterile distilled water.

We selected 31 *Eco*RI and *Msp*I/*Hpa*II primer combinations for selective PCRs (Table 2). The amplification reaction was performed in a total volume of 20 µl with 3.75 mmol dNTP (with Mg^{2+}), 5 pmol of fluorescently labeled (6-FAM) forward primer, 5 pmol of reverse primer, 1.25 U of *Taq* DNA polymerase, and

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Table 5. Percentage of three methylation states.

Population/region/species[a]		No. of samples	Un-methylated	Hemi-methylated	Full-methylated
DG	DG2	15	18.58%	36.02%	45.40%
	DG3	15	20.24%	36.33%	43.43%
	DG4	15	20.22%	37.84%	41.94%
	Average	15	19.68%	36.73%	43.59%
	DG	45	19.86%	35.37%	44.76%
HK	HK1	15	15.70%	58.25%	26.05%
	HK3	15	16.61%	51.80%	31.59%
	HK4	15	18.23%	57.03%	24.74%
	HK5	10	14.96%	59.08%	25.96%
	HK6	15	26.11%	36.90%	36.99%
	HK7	11	23.54%	36.34%	40.12%
	HK8	10	26.51%	37.31%	36.18%
	Average	13	20.24%	48.10%	31.66%
	HK	91	20.07%	47.25%	32.68%
MA	MA1	15	12.28%	68.33%	19.40%
	MA4	16	17.43%	60.60%	21.97%
	Average	15.5	14.86%	64.47%	20.69%
	MA	31	15.04%	62.85%	22.11%
NLD	NLD2	15	18.37%	34.47%	47.16%
	NLD3	16	18.02%	39.82%	42.15%
	NLD5	15	18.75%	40.40%	40.85%
	NLD6	16	19.89%	40.62%	39.50%
	Average	15.5	18.76%	38.83%	42.42%
	NLD	62	19.16%	37.32%	43.51%
SZ	SZ1	16	21.77%	37.20%	41.02%
	SZ4	15	19.22%	33.89%	46.89%
	SZ5	15	18.32%	34.91%	46.78%
	Average	15.3	19.77%	35.33%	44.90%
	SZ	46	19.37%	34.23%	46.40%
ZH	ZH1	15	20.25%	40.06%	39.69%
	ZH2	16	19.57%	40.89%	39.54%
	Average	15.5	19.91%	40.48%	39.62%
	ZH	31	20.25%	40.00%	39.75%
Total		306	19.23%	41.69%	39.09%

[a]See Table 1 for codes.

2.5 µl of diluted preselective PCR product. The PCR profile was as follows: 94 C for 5 min, 13 touchdown cycles of 94 C for 30 s, 65 C for 30 s reduced by 0.7 C per cycle, and 72 C for 1 min; 23 cycles of 94 C for 30 s, 56 C for 30 s, and 72 C for 1 min, and a final elongation step at 72 C for 7 min. Selective PCR products were separated and visualized on an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA) with internal size standard LIZ 500 (Figure 2). Final profiles were analyzed using GeneMarker v. 2.2.0 software (SoftGenetics, State College, PA, USA), and translated into a presence (1)/absence (0) data matrix. To minimize the potential impact of size homoplasy (Caballero et al. 2008), we selected fragments ranging from 150 bp to 500 bp. A peak height threshold was set as 1,000 to 30,000. Furthermore, to ensure low error rates, an E4-H/M3 primer pair was used to assess the reproducibility of the MSAP assay in 306 individuals. Error rate was estimated as only 0.027%.

Methylation Scoring

As isoschizomers, *Hpa*II and *Msp*I cleave the same sequence (CCGG) with subtle differences. The former only recognizes methylated external cytosine on a single strand, while the latter recognizes methylated internal cytosine on a single or both strands. Hence, four methylation types can be obtained: Type I denotes no methylation due to cleavage by both enzymes; Type II denotes full-/hemi-methylation of internal cytosine only when *Msp*I cuts; Type III denotes hemi-methylation of external cytosine only when *Hpa*II cuts; and Type IV is free of any enzyme cutting, possibly due to full methylation of external cytosine, full methylation of both cytosines, hemi-methylation of both cytosines, or a restriction site mutation. In this study, Type IV was considered to be uninformative, because it could be attributed to multiple and equivocal reasons.

Three scoring approaches were adopted for our data (Schulz et al. 2013): (1) *Eco*RI/*Msp*I data were treated using the methylation-sensitive (MS)-AFLP "10" matrix or genetic matrix; (2) Type II and Type III were considered to be methylated loci "1," while the other two types were marked as "0," which we called the "Salmon matrix" or "Salmon Scoring" (Salmon et al. 2008); (3) a "Mixing Scoring 2" method was used to obtain four different matrices: H, M, U, and HMU matrix. In matrix H, only Type III was considered as "1"; in matrix M, only Type II was considered as "1"; in matrix U, only Type I was regarded as "1"; and matrix HMU included H, M, and U in order (Table 3). All our analyses proceeded based on these six matrices.

Soil Chemical Analyses

Soil samples collected from 21 *M. micrantha* invasive populations were air-dried for 14 d and ground to pass through a sieve with an aperture size of 1 mm and 0.2 mm, respectively. Water contents of fresh and air-dried soil were determined by oven-drying for 6 h at

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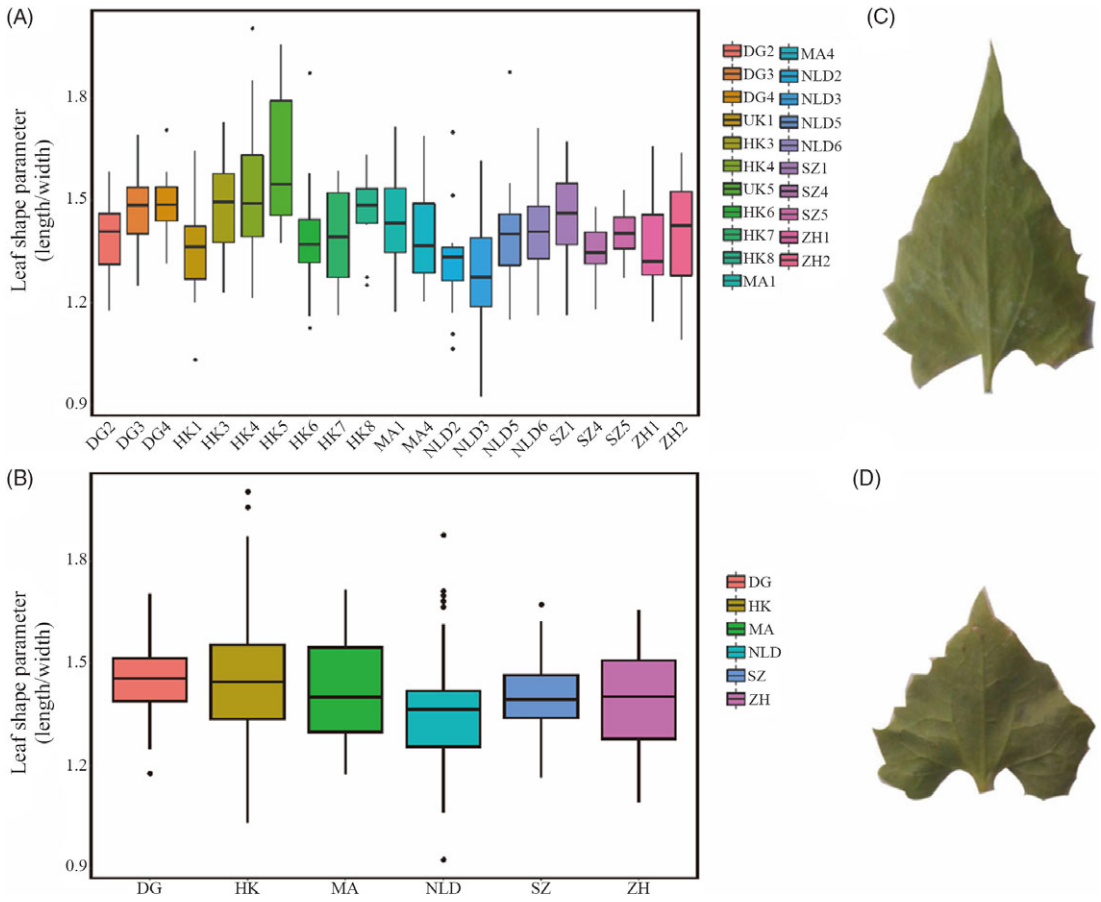

Figure 3. Leaf shape of *Mikania micrantha* varied by population and region. (A) Leaf shape parameter in 21 populations. (B) Leaf shape variation in six regions. (C) A high leaf length:width ratio is represented using individual 6 of the HK5 population. (D) A low leaf length:width ratio is represented using individual 11 of the NLD3 population. See Table 1 for population codes.

105 C. Soil pH and electrical conductivity were measured in a solution of soil mixed with water at a ratio of 200 g L^{-1} using a pH meter (DPS-307A, INESA, Shanghai, China) and a conductivity meter (DPS-307A, INESA, Shanghai, China), respectively. Soil organic matter content was measured by using the potassium dichromate volumetry method. Soil total nitrogen was quantified by the Kjeldahl method using a Kjeltec^{TM} 8400 Analyzer Unit (Foss, Hillerod, Denmark) after digestion at a ratio of 100 g L^{-1} soil to H_2SO_4. Total carbon was determined in a total organic carbon analyzer (Shimadzu, Kyoto, Japan) at 720 C. Soil K, Ca, Na, Mg, Al, P, S, Si, Fe, Mn, Zn, Cu, Pb, Cr, As, Se, Ni, and Cd were assayed using an inductively coupled plasma optical emission spectrometer (ICP-OES, PerkinElmer, Waltham, MA, USA) after digestion with HNO_3/HCl/HF at a ratio of 3:1:1 (v/v/v) using a MARS 6 Microwave Reaction System (CEM, Matthews, NC, USA). Experimental treatments without soil samples served as a control. All measurements were repeated three times.

Data Analysis

GenAlEx v. 6.41 (Peakall and Smouse, 2012) was used to assess (epi)genetic parameters, including observed number of alleles (N_a), effective number of alleles (N_e), Shannon's diversity index (I), number of private bands, percentage of polymorphic loci (%P), expected heterozygosity (H_e), and unbiased expected heterozygosity (UH_e). We also calculated pairwise geographic distance and (epi) genetic distance matrices between populations.

Analysis of molecular variance (AMOVA) was carried out using Arlequin v. 3.5 (Excoffier and Lischer 2010) to estimate MS-AFLP genetic and epigenetic variation partitioned among groups of populations (F_{ct}), among populations within groups (F_{sc}), and among populations (F_{st}) with 10,000 permutations. A Mantel test was performed to explore linear correlation between geographic distance and (epi)genetic distance. A partial Mantel test was used to detect correlation between MS-AFLP genetic matrix and different epigenetic matrices. Significance level was evaluated using 10,000 permutations (Smouse et al. 1986).

We assessed (epi)genetic structure by using Structure v. 2.3.4 (Falush et al. 2007) to allocate individuals into clusters based on a Bayesian clustering method. The best K value was determined according to Ln P (D), which is an estimate of the posterior probability of the data for a given K, and ΔK. Principal coordinate analysis (PCoA) based on Dice's distance matrices (Zoldoš et al. 2018) was performed to visualize the population relationship using PAST v. 3.18 (Hammer et al. 2011). Sequentially, the "10" data set was aligned in MEGA v. 6.0 and further converted to a nexus format (Tamura et al. 2013). An UPGMA tree was constructed using PAUP v. 4.0 with 1,000 permutations (Swofford 2001). Candidate adaptive loci were identified using Dfdist (Beaumont and Nichols

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Table 6. Results of analysis of molecular variance (AMOVA).

Variation source	df	Variation percentage	Differentiation value
AFLP			
Among populations within groups	5	10.39%	$F_{sc} = 0.1005$
Among populations	15	9.08%	$F_{st} = 0.1939$
Among groups	285	9.00%	$F_{ct} = 0.1039$
Salmon			
Among populations within groups	5	6.85%	$F_{sc} = 0.0719$
Among populations	15	6.73%	$F_{st} = 0.1355$
Among groups	285	6.70%	$F_{ct} = 0.0685$
H			
Among populations within groups	5	5.34%	$F_{sc} = 0.0734$
Among populations	15	6.95%	$F_{st} = 0.1229$
Among groups	285	6.95%	$F_{ct} = 0.0534$
M			
Among populations within groups	5	10.07%	$F_{sc} = 0.0844$
Among populations	15	7.68%	$F_{st} = 0.1766$
Among groups	285	7.59%	$F_{ct} = 0.1007$
U			
Among populations within groups	5	6.66%	$F_{sc} = 0.0885$
Among populations	15	8.22%	$F_{st} = 0.1492$
Among groups	285	8.26%	$F_{ct} = 0.0666$
HMU			
Among populations within groups	5	7.27%	$F_{sc} = 0.0801$
Among populations	15	7.45%	$F_{st} = 0.1470$
Among groups	285	7.43%	$F_{ct} = 0.0727$

[a]F_{sc}, (epi)genetic variation among populations within groups; F_{st}, (epi)genetic variation among populations; F_{ct}, (epi)genetic variation among groups.

Table 7. F_{st} value from six matrices at regional and species levels.[a]

	AFLP	Salmon	H	M	U	HMU
DG	0.0593	0.0404	0.0339	0.0502	0.0489	0.0435
HK	0.1292	0.0838	0.0856	0.1128	0.1062	0.0979
MA	0.0940	0.0748	0.0735	0.0707	0.0968	0.0769
NLD	0.0941	0.0688	0.0671	0.0774	0.0803	0.0736
SZ	0.1016	0.0891	0.0946	0.0892	0.0929	0.0921
ZH	0.0250	0.0211	0.0192	0.0317	0.0185	0.0235
Total	0.1808	0.1263	0.1156	0.1637	0.1404	0.1374

[a]DG, Dongguan; HK, Hong Kong; MA, Macao; NLD, Nei Lingding Island; SZ, Shenzhen; ZH, Zhuhai.F_{st}, (epi)genetic variation among populations.

1996) and BayeScan 2.1 (Foll and Gaggiotti 2008). The former was conducted to detect outliers with 50,000 simulations with a 99.5% confidence interval (CI), while the latter was run with a sample size of 5,000, a thinning interval of 20, and 10 pilot runs of 5,000 iterations for burn-in. Only loci that were simultaneously detected by Dfdist and with a strong detection level (posterior odds, PO ≥ 100) in BayeScan were considered as candidate adaptive loci. Samβada v. 0.4.5 (http://lasig.epfl.ch/sambada; Joost et al. 2007; Stucki et al. 2017) was adopted to identify candidate loci that may play important roles in response to changing environments. To ensure accuracy, we also applied the spatial analysis method (SAM) to identify the correlation of candidate loci and environmental factors, including climatic and soil variables based on multiple univariate logistic regression (Joost et al. 2007). Climatic data were downloaded from WorldClim v. 2 (http://www.worldclim.org) and extracted by ArcGis software. Soil data were generated in our lab (see above). Linkage disequilibrium (LD) among adaptive loci was detected using TASSEL software (Bradbury et al. 2007) with criteria of $R^2 > 0.3$ and $P < 0.001$ (Keller et al. 2012).

In addition, SAM (Joost et al. 2007) was also used to analyze the large-scale spatial (epi)genetic structure. Shannon's diversity index (*I*) was used for population diversity data. Distances were divided into five classes according to the maximum actual distance between two populations (85 km). SAM analyses were conducted with 9,999 permutations and 95% CI. The fine-scale spatial (epi)genetic structure was assessed by linear regression of pairwise relationship coefficients for 10 distance classes using SPAGeDi v. 1.3 (Hardy and Vekemans 2002).

We collected geographic information such as longitude and latitude of *M. micrantha* in southern China from the literature, the Global Biodiversity Information Facility (https://www.gbif.org), the National Specimen Information Infrastructure (http://www.

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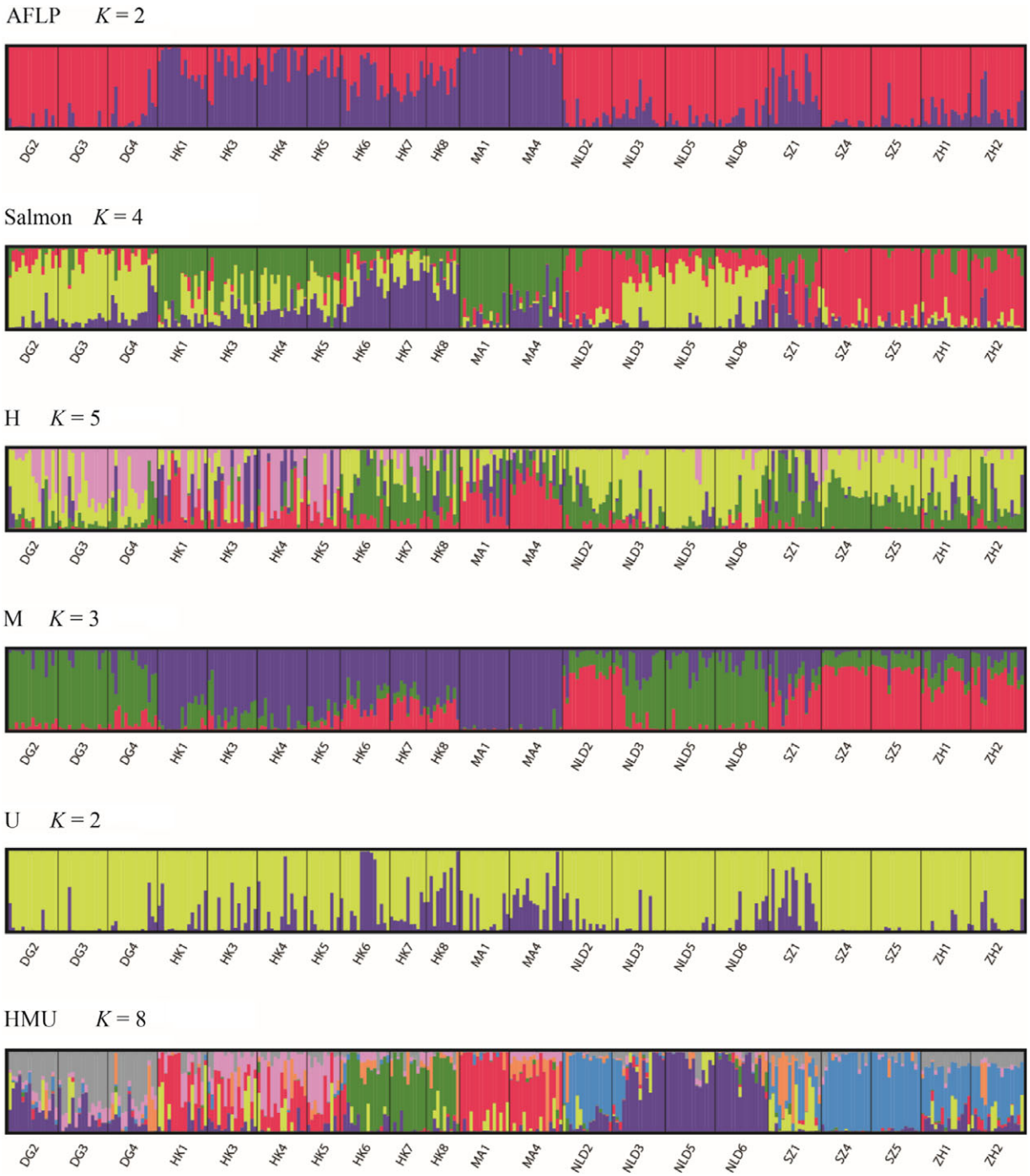

Figure 4. STRUCTURE results with different *K* values for the six matrices. See Table 1 for population codes.

nsii.org.cn), and the Chinese Virtual Herbarium (http://www.cvh.ac.cn). After removal of duplicate locations, we obtained 298 distribution sites for niche model prediction. Maxent software (Phillips et al. 2006) was used to predict current and future distribution of *M. micrantha* with 10 cross-validated replicated runs, of which the latter proceeded based on the HadGEM2-ES and RCP4.5 model (Collins et al. 2011; Thomson et al. 2011). Area under the curve (AUC) was used to assess model performance. AUC values > 0.9 are usually taken as an indicator of high-accuracy models (Swets 1988) and efficient model performance (Manel et al. 2001).

Sequencing of Methylated Polymorphic Fragments

After 6% denaturing polyacrylamide gel electrophoresis and silver staining for visualization, clear and methylated bands were extracted, purified, and sequenced (Zhou and Wang 2013)

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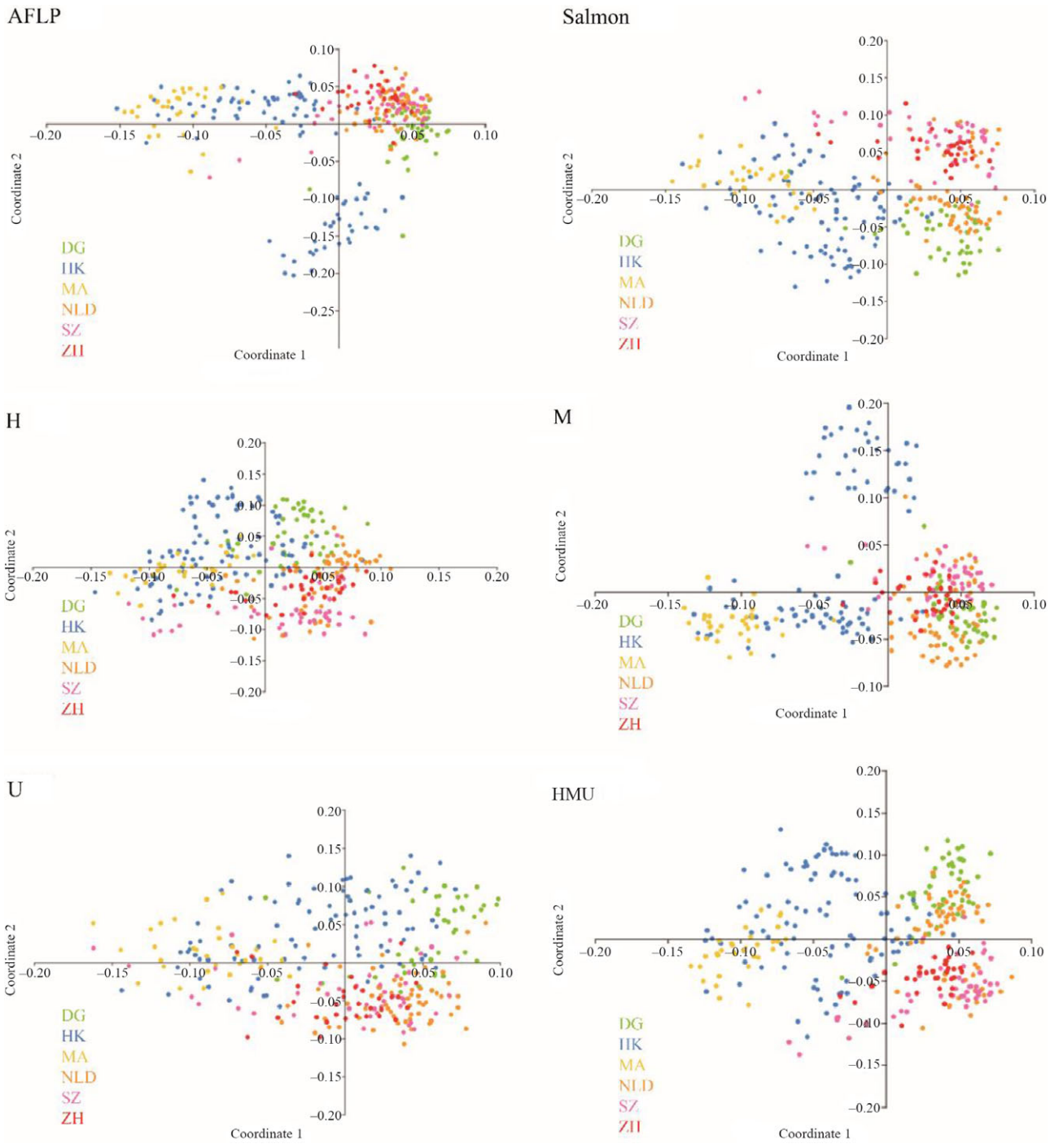

Figure 5. Principal coordinate analysis (PCoA) results for the six matrices. See Table 1 for population codes.

(Guangzhou Tsingke Biotechnology, Ltd., Guangzhhou, Guangdong, China). Their sequences were BLAST searched against the NCBI and Ensembl databases.

Results and Discussion

Impacts of MSAP Scoring Approaches on (Epi)genetic Variation Estimates

This is the first report on effects of environmental factors on population epigenetic differentiation in *M. micrantha* using MSAP. So far, there exists no consensus method for scoring the information generated from the MSAP banding patterns. As for the six matrices we generated, except for the MS-AFLP genetic matrix, all other matrices were treated as epigenetic data. Of note, Mixing Score 2 included matrices H, M, and U, which were further combined into matrix HMU. Descriptive (epi)genetic parameters were then estimated for all six matrices at the population, regional, and species levels (Table 4; Supplementary Table S1). For each matrix, differences were found for such (epi)genetic parameters as the number of alleles and effective alleles, Shannon information index (*I*), percentage of polymorphic loci (%*P*), expected heterozygosity

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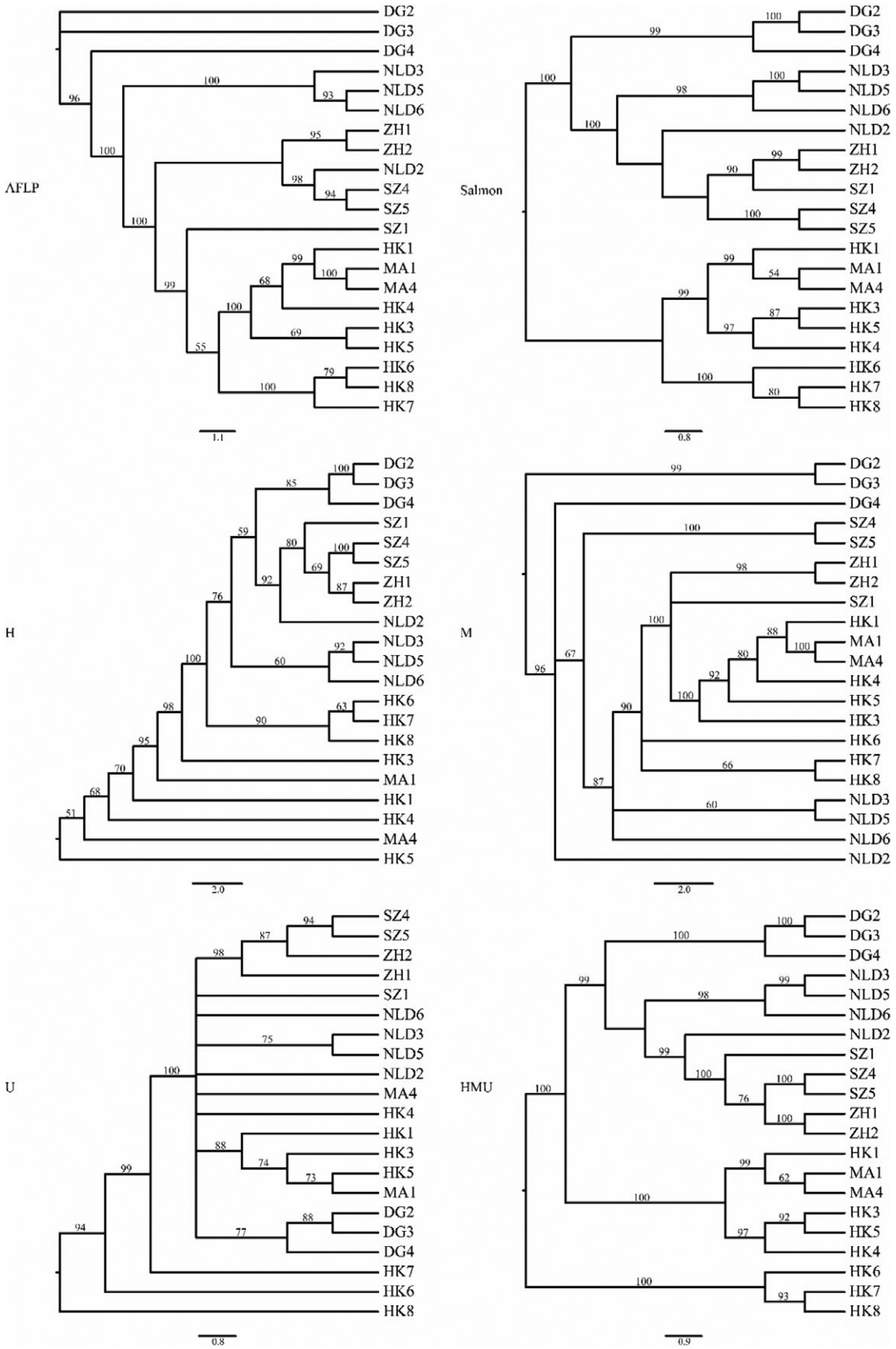

Figure 6. UPGMA tree computed using PAUP based on the six matrices. The number on the branch indicates the bootstrap value. See Table 1 for population codes.

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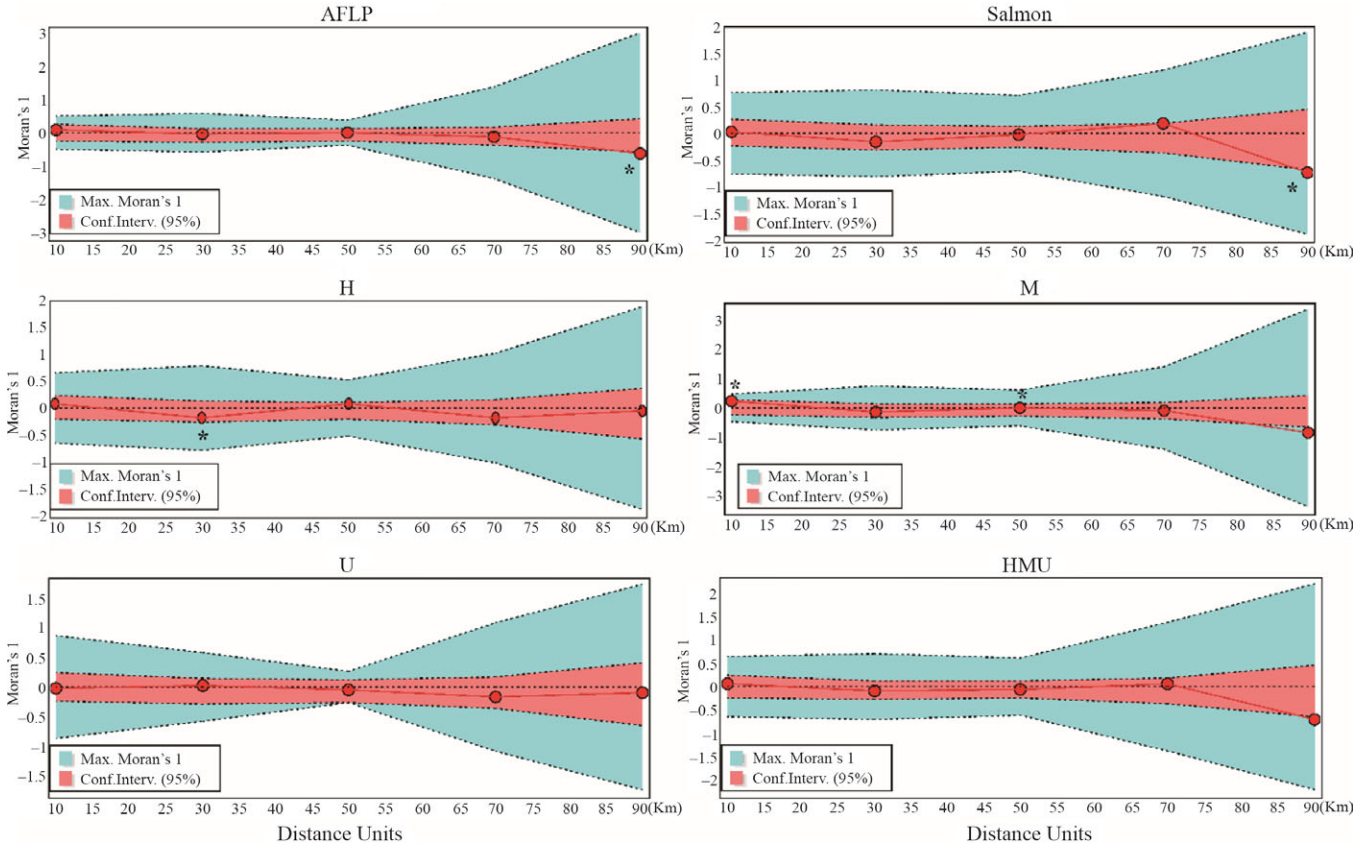

Figure 7. Large-scale spatial structure with five distance classes based on the six matrices. An asterisk (*) indicates significance level ($P < 0.05$).

(H_e), and unbiased expected heterozygosity (UH_e) (Friedman test, $P < 0.05$). At the species level, the highest (epi)genetic diversity occurred in the Salmon matrix, while the lowest was in the U matrix. Our results are consistent with Schulz et al.'s (2013) findings that scoring schemes had strong effects on the estimates of epigenetic diversity and differentiation. In this study, we adopted both Salmon Scoring and Mixing Scoring 2 to evaluate the epigenetic variation of *M. micrantha*. In comparison to Salmon Scoring, Mixing Scoring 2 incorporates scoring for both methylated and unmethylated bands, utilizing more of the underlying banding pattern information. Nevertheless, it needs to be noted that there does not seem to be one best method for scoring MSAP bands for multilocus analyses (Schulz et al. 2013).

MS-AFLP genetic matrix was used to survey genetic diversity (Foust et al. 2016; Schulz et al. 2013). We detected 1,800 polymorphic MS-AFLP genetic loci, whose percentage (%*P*) ranged from 16.17% to 40.5%. The Shannon diversity index (*I*) varied from 0.052 to 0.137, while the expected heterozygosity (H_e) ranged from 0.031 to 0.083. These results are not quite consistent with those obtained by using actual AFLP to assess *M. micrantha* (Wang et al. 2012). Similar instances have been observed in South African ragwort (*Senecio inaequidens* DC) (Lachmuth et al. 2010; Monty et al. 2013). This is not unexpected, considering that MSAP is technically a modification of AFLP.

We also evaluated epigenetic parameters. Based on the Salmon matrix, 2,523 polymorphic loci were identified with percentages of 31.11% to 41.78%; *I* and H_e values ranged from 0.110 to 0.138 and 0.066 to 0.084, respectively. Using the H matrix, 2,260 were identified as polymorphic loci with percentages of 20.49% to 38.1%; *I* valued from 0.06 to 0.109, and H_e from 0.035 to 0.063. For the M matrix, 1,487 polymorphic loci were detected with percentages of 13.45% to 39%; I varied from 0.042 to 0.128, and H_e from 0.025 to 0.078. For the U matrix, 1,239 polymorphic loci were detected with percentages of 12.5% to 33.82%; *I* ranged from 0.035 to 0.094, and H_e from 0.02 to 0.055. The HMU matrix produced a total of 4,966 loci, with %*P* from 21.1% to 33.33%, *I* from 0.002 to 0.092, and H_e from 0.002 to 0.054.

Patterns of Population (Epi)genetic Variation and the Association of Epigenetic Variation with Leaf Plasticity

In this study, a total of 2,840 scorable polymorphic loci were obtained using 31 pairs of selective PCR primers across 306 individuals. Overall, the percentage of hemi-methylated loci ranged from 33.89% (SZ4) to 68.33% (MA1), with an average of 41.69%, followed by fully methylated loci from 19.40% (MA1) to 46.89% (SZ4), with an average of 39.09%. By contrast, the percentage of unmethylated loci ranged from 12.28% (MA1) to 26.51% (HK8), with an average of 19.23% (Table 5). Hong Kong populations exhibited the lowest level of full methylation.

Twenty-one populations were found to have three different methylation levels (*t*-test, $P < 0.05$), and different methylation levels were also detected at regional levels. Total methylation (full and hemi-methylation) was different between Macao and Hong Kong and the other four regions. For hemi- or full methylation, Macao, Hong Kong, and Zhuhai each were found to be different from the

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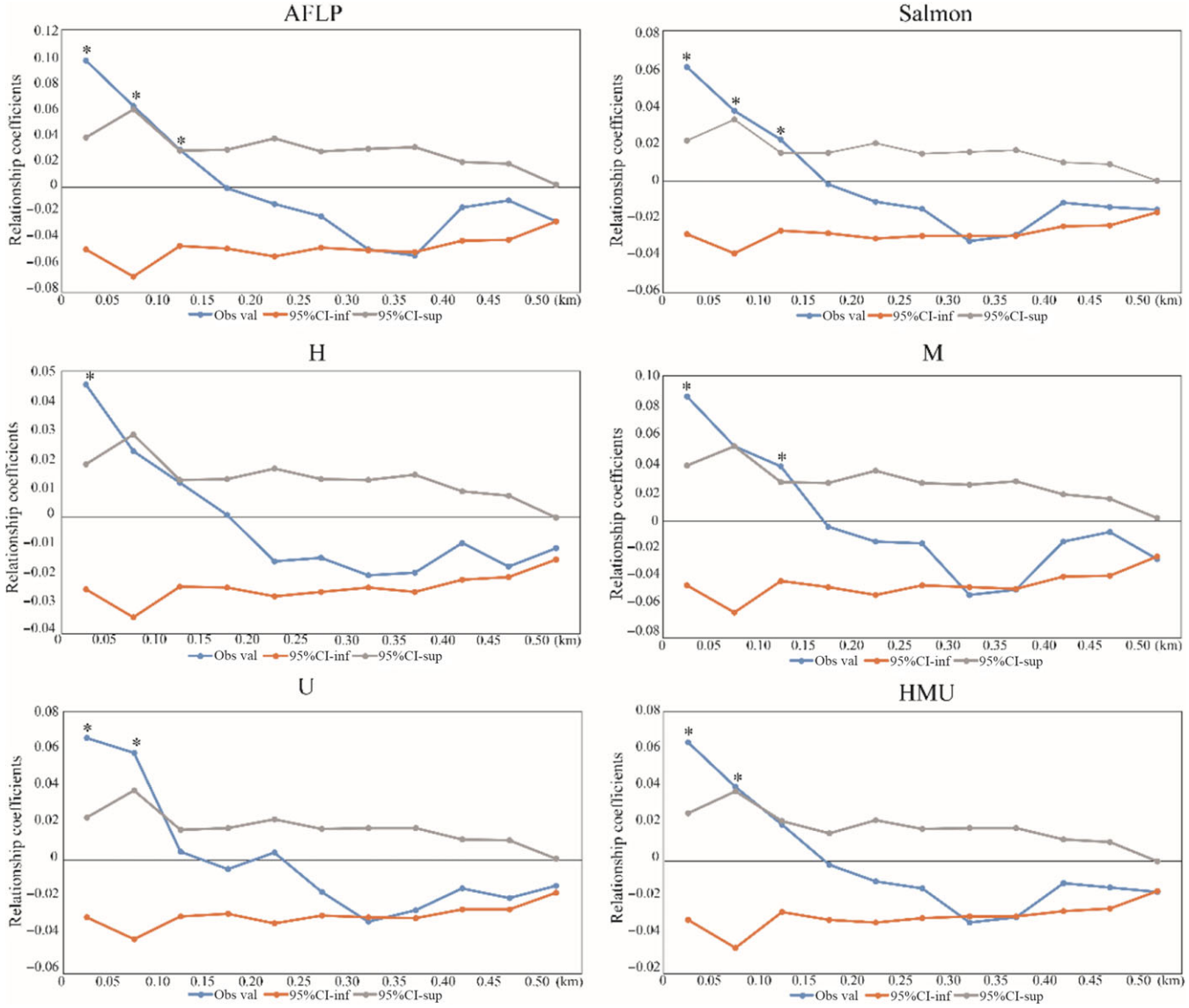

Figure 8. Fine-scale spatial structure at 10 distance classes based on six matrices. An asterisk (*) indicates significance level ($P < 0.05$).

other regions. Regarding hemi-methylation, a significant difference was detected between Nei Lingding and Zhuhai.

Mikania micrantha had the highest epigenetic diversity at the species level based on Salmon Scoring, followed by MS-AFLP genetic variation, implying that methylation variation plays a role in its invasive evolution. The genetic variation of *M. micrantha* is lower (0.08) in comparison to other invasive plants like lantana (*Lantana camara* L.) (0.28), annual bluegrass (*Poa annua* L.) (0.17), maritime pine (*Pinus pinaster* Aiton) (0.15) (Blignaut et al. 2013), *S. inaequidens* (0.30) (Monty et al. 2013), and smooth cordgrass (*Spartina alterniflora* Loisel.) (0.38) (Foust et al. 2016), based on the same molecular marker. Importantly, *M. micrantha* also has lower epigenetic variation (0.04 to 0.08) than another invasive plant, *S. alterniflora* (0.37) (Foust et al. 2016). In addition, it also maintains lower population-level genetic differentiation compared with other invasive Asteraceae plants in China like Jack in the bush [*Chromolaena odorata* (L.) R.M. King & H. Rob.] and Santa Maria feverfew (*Parthenium hysterophorus* L.) (Ma et al. 2011; Tang et al. 2009; Ye et al. 2004). The low level of population-level genetic differentiation of *M. micrantha* might be related to its clonal reproduction and relatively short invasive history in China.

Compared with noninvasive species, invasive species tend to display higher phenotypic plasticity (Davidson et al. 2011). In this study, the length:width ratio was used to characterize leaf shape and evaluate leaf plasticity (Figure 3). Leaf shape was found to exhibit considerable difference among populations. In particular, leaf shape in Nei Lingding was much different from that in Dongguan, Hong Kong, and Macao. Pearson analysis showed that leaf shape was significantly related to H matrix–based N_e ($r^2 = 0.502$), H_e ($r^2 = 0.468$), and UH_e ($r^2 = 0.497$) and the full-methylation level ($r^2 = -0.433$) at the population level; and I ($r^2 = 0.865$) and UH_e ($r^2 = 0.816$) at the regional level ($P < 0.05$). These results imply that the leaf shape variation of *M. micrantha* may be linked to methylation-state changes. Similarly, Gao et al. (2010) described the correlation between phenotypic variation and methylation alternations in the invasive weed alligatorweed [*Alternanthera philoxeroides* (Mart.) Griseb.].

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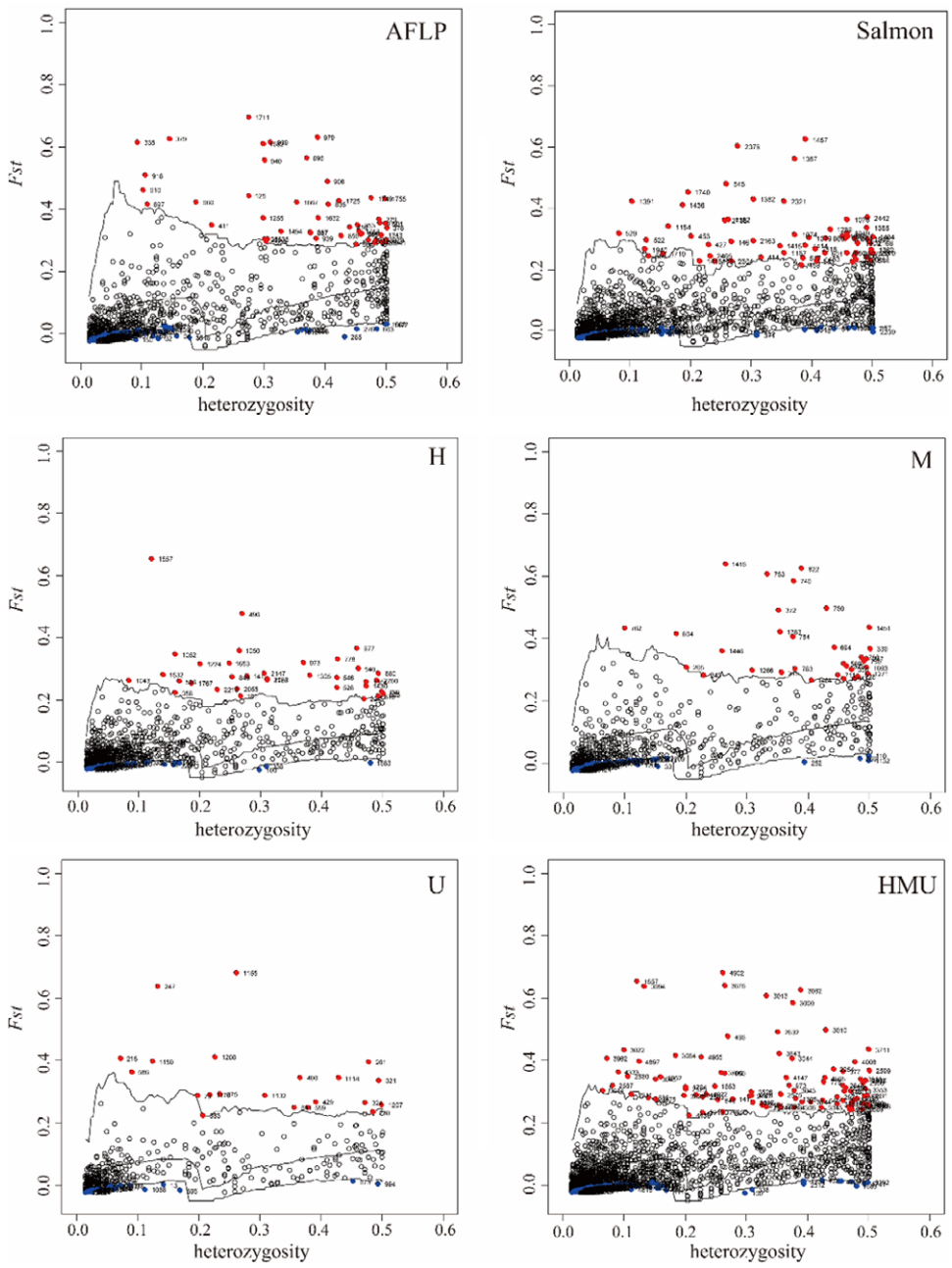

Figure 9. Outliers were identified by Dfdist analysis. The red dots indicate positively selective loci.

Population (Epi)genetic Differentiation among Regions

Based on regions, the 21 populations were divided into six groups, and AMOVA was performed (Table 6). Populations exhibited higher levels of epigenetic differentiation than genetic differentiation. F_{st} values among Hong Kong populations were found to be significantly higher than those among the Dongguan, Macao, Nei Lingding, and Zhuhai populations, but similar to those of Shenzhen populations ($P < 0.05$; Table 7).

Based on the genetic matrix, intrapopulation and interpopulation variation accounted for 80.61% and 19.39% of the total variation, respectively. As for the epigenetic Salmon matrix, 86.45% and 13.55% of the variation resided within and among populations separately. When using the H, M, U, and HMU matrices, 87.72%, 82.34%, 85.08%, and 85.30% of the variation were partitioned within populations, while 12.29%, 17.66%, 14.92%, and 14.70% were among populations, respectively.

Genetic differentiation ($F_{st} = 0.1939$) was higher in comparison to epigenetic differentiation (F_{st}: 0.1229 to 0.1766; Table 6). A Mantel test was performed based on Nei's genetic distance and geographic distance between populations. Except for the U matrix, most matrices were found to have a significant correlation between geographic and (epi)genetic distance ($P < 0.05$). Among them, the highest correlation occurred with the Salmon matrix ($r^2 = 0.235$), while the lowest occurred with the M matrix ($r^2 = 0.178$). It is of interest to note that no clear geographic genetic structure was detected among the roadside populations of *M. micrantha* in

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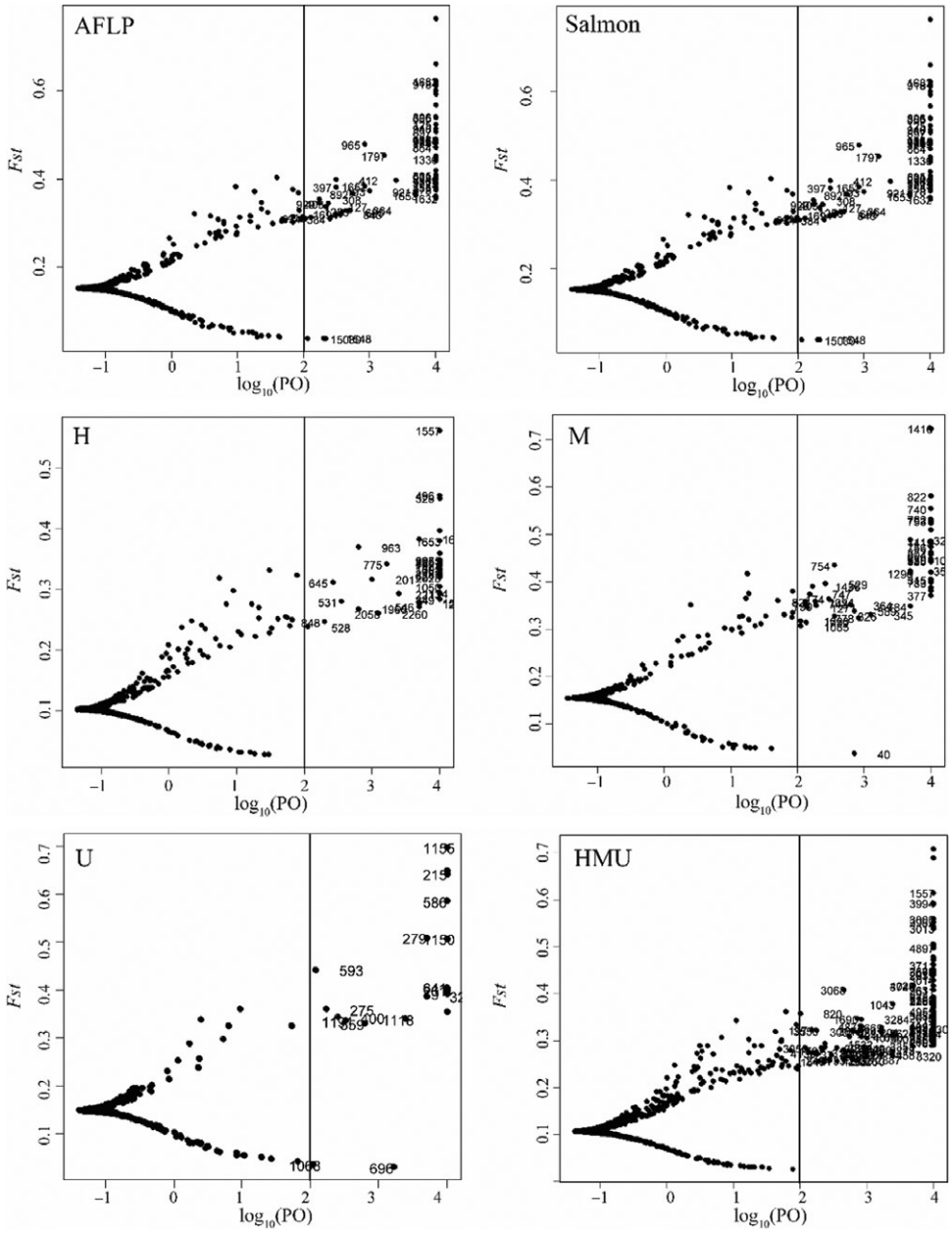

Figure 10. Candidate positively selective loci were detected by Bayescan analysis. Decisive selected loci were determined according to ln (PO) ≥ 2.

southern China when using microsatellite markers (Geng et al. 2017).

The relationship between the genetic and five epigenetic matrices was further investigated. The correlation index was 0.8951, 0.4812, 0.9404, 0.7528, and 0.9192 for the Salmon, H, M, U, and HMU matrices, respectively, indicating a high correlation between genetic and epigenetic variation.

Investigation of Spatial (Epi)genetic Structure

We found that there was a clear (epi)genetic structure in genetic, Salmon, and M matrix (Figure 4). Based on the genetic matrix, the Hong Kong and Macao populations formed one cluster, while the remaining populations formed another. Comparatively, epigenetic structure was more complex (Figure 4). Different epigenetic matrices yielded distinct clustering results, indicating the potential effects of methylation states on population structure. Based on the M matrix, the Hong Kong and Macao populations also formed one cluster; three Nei Lingding populations (NLD3, NLD5, and NLD6) clustered into one group together with Dongguan populations, while one Nei Lingding population (NLD2) clustered into another group with Shenzhen and Zhuhai populations. The epigenetic structure constructed from the Salmon matrix was more subtly complicated. The structure between the four Nei Lingding populations and the other populations was similar to that from the M matrix, with the main difference seen with the Hong Kong populations; three Hong Kong populations (HK6, HK7, and HK8) clustered together, while the other four (HK1, HK3, HK4, and HK5) grouped with the Macao populations. As for the other three matrices, H, U, and HMU, no clear epigenetic structure was detected.

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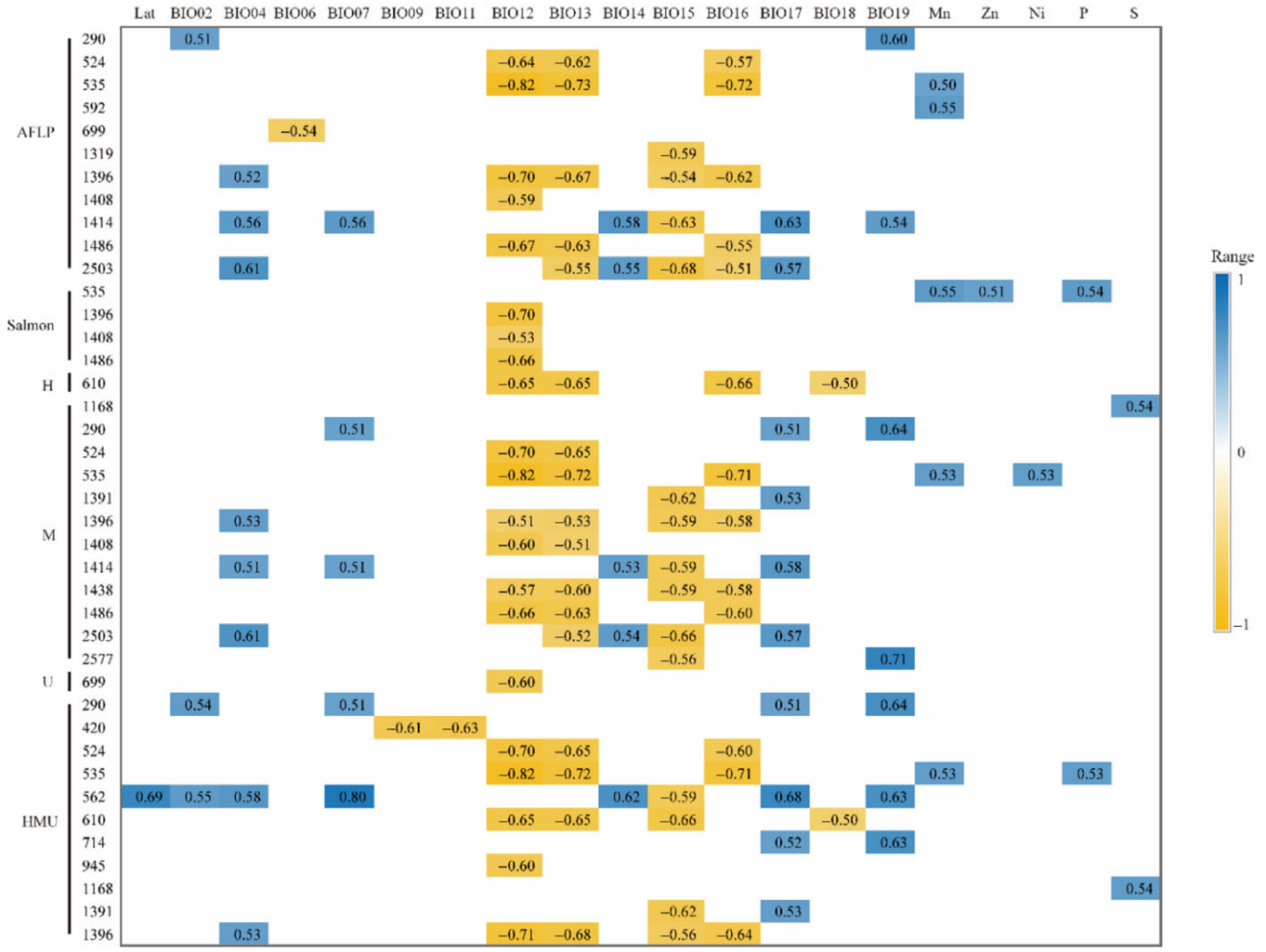

Figure 11. Correlation was investigated between adaptive loci and environmental factors. The correlation range was from −1 to 1. Positive and negative values indicated that loci were positively and negatively correlated with the environmental factors, respectively. The greater the absolute value, the stronger the correlation, and vice versa.

PCoA and UPGMA revealed similar results (Figures 5 and 6), with subtle differences when using the HMU matrix, in which the Hong Kong populations (HK1, HK3, HK4, and HK5) grouped with the Macao populations in the UPGMA tree (Figure 6). These results suggested that there existed a certain degree of gene flow between the populations and highlighted the importance of Hong Kong populations in the invasion of *M. micrantha* in southern China; the Hong Kong and Macao populations tend to cluster together based on either genetic or epigenetic data. This may be related to historical reasons, as Hong Kong had more trade exchange with Macao than mainland China in the 1980s, which possibly contributed to the population mixture (Wang et al. 2003; Zhang et al. 2004).

We further examined spatial (epi)genetic structure at two scales. At the large scale, for the genetic matrix, a significant negative Moran's *I* value (−0.272, P = 0.035) was observed for the 80- to 100-km distance class. Similarly, for the Salmon matrix, a significant negative *I* value (−0.743, P = 0.021) was returned for the same distance class. For the H matrix, a significant negative *I* value (−0.29, P = 0.035) was detected in the 20- to 40-km distance class. Base on the M matrix, a significant positive *I* value (0.265, P = 0.047) was detected in the 0- to 20-km distance class, while a significant negative *I* value (−0.341, P = 0.028) was observed in the 40- to 60-km distance class. No significant value was detected for *I* for any distance classes based on the U and HMU matrices (Figure 7).

At the fine scale, the spatial structure was examined in 50-m intervals (Figure 8). For the genetic matrix, a significant positive autocorrelation was found in the first three distance classes (0 to 50 m, 50 to 100 m, and 100 to 150 m; P < 0.05). For the Salmon matrix, a similar spatial structure was detected. Using the U and HMU matrices, significant positive autocorrelation was found in the first two distance classes (0 to 50 m and 50 to 100 m; P < 0.05). For the M matrix, significant positive autocorrelation was detected in the first and third distance classes (0 to 50 m and 100 to 150 m; P < 0.05). And for the H matrix, the spatial structure was observed only in the first distance class (0 to 50 m, P < 0.05).

Identification of Candidate Selective Loci and Local Adaptive Response to Novel Environment

We detected adaptive loci and epiloci. Using Dfdist, 47 and 151 loci were identified with positive and negative selection in the genetic

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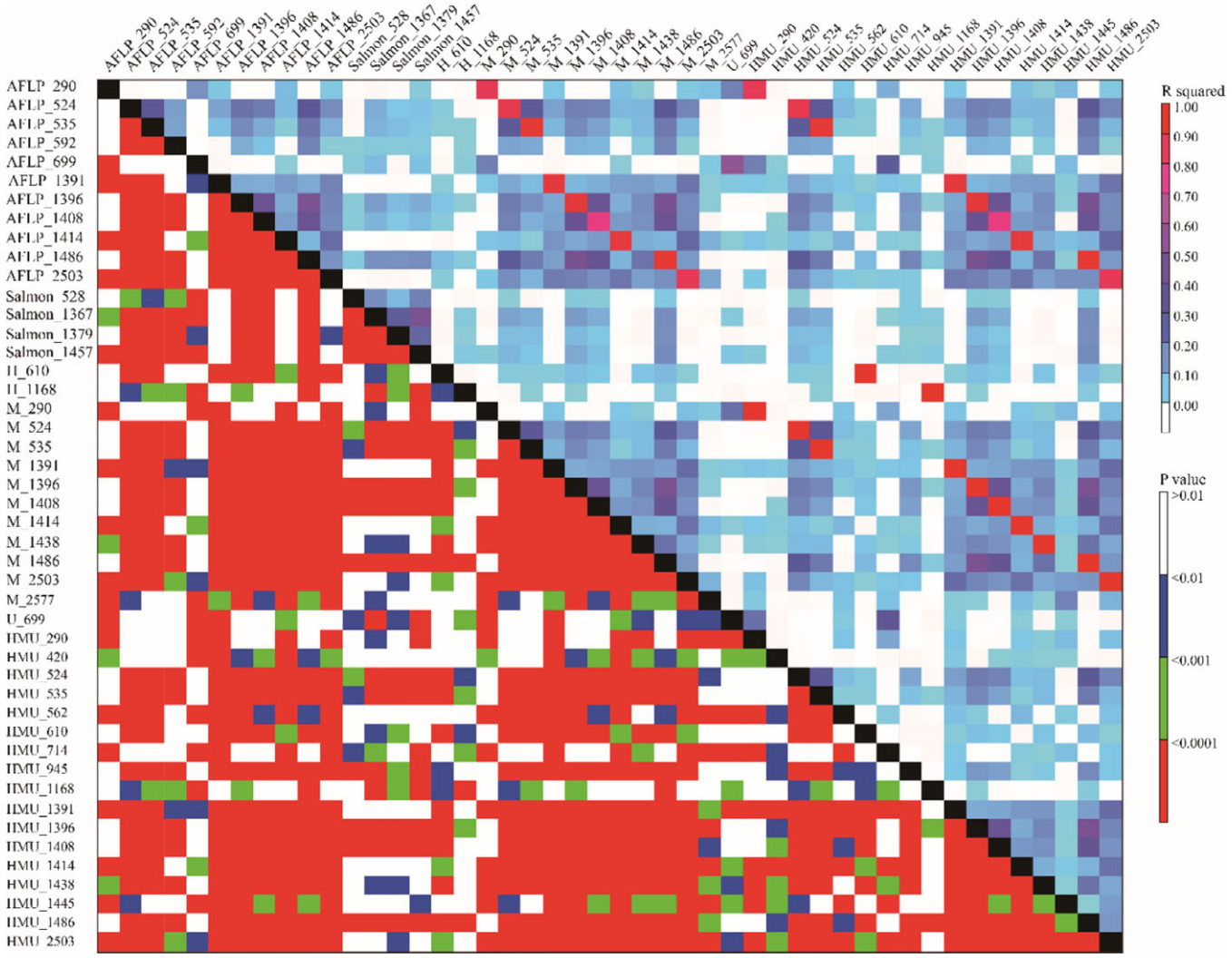

Figure 12. A linkage disequilibrium (LD) test was conducted among adaptive loci. Areas above and below the diagonal indicate the R^2 and P-value, respectively.

matrix, respectively. By contrast, 61 and 118 loci were detected under positive and negative selection for the Salmon matrix, 34 and 63 for the H matrix, 32 and 100 for the M matrix, 21 and 37 for the U matrix, and 93 and 226 for the HMU matrix, respectively (Figure 9).

Using Bayescan, 66 and 1 loci were found to be under positive and negative selection in the genetic matrix, respectively. Comparatively, the corresponding loci were 87 and 1 for the Salmon matrix, 38 and 1 for the H matrix, 48 and 1 for the M matrix, 20 and 1 for the U matrix, and 124 and 1 for the HMU matrix, respectively (Figure 10). To ensure accuracy, only those loci that had been simultaneously identified by both Dfdist and Bayescan were finally considered as being under selection. As a result, we found 39 candidate selective loci in the genetic matrix, 56 for the Salmon matrix, 22 for the H matrix, 27 for the M matrix,15 for the U matrix, and 81 for the HMU matrix, respectively.

We used Samβada software to detect locus–environmental variable associations. It identified 182, 122, 13, 136, 37, and 199 candidate loci for the genetic, Salmon, H, M, U, and HMU matrices, respectively. The majority of these candidate loci were associated with multiple environmental variables. The majority of these candidate loci were further subjected to univariate linear regression model analysis. Only loci with $R^2 \geq 0.5$ were considered as adaptive (Figure 11), which was 11, 4, 2, 11, 1, and 17 for the genetic, Salmon, H, M, U, and HMU matrices, respectively. Among them, five loci were simultaneously detected in three matrices, and four were detected in four matrices. In total, for the six matrices, 21 adaptive loci were detected that were positively correlated with such environmental factors as soil Mn, Zn, Ni, P, and S; latitude; mean diurnal range; temperature seasonality; temperature annual range; precipitation of driest month; precipitation of driest quarter; and precipitation of coldest quarter. Of note, there were more adaptive epiloci (20) than adaptive loci (11) related to environmental factors. Moreover, LD was found in the adaptive (epi)loci (Figure 12). Our results underscore the relative importance of methylation-based epigenetic variation in the adaptive response to environmental conditions (Herrera and Bazaga 2010). Also of note, no adaptive (epi)loci were found to be solely linked to temperature, but some were simultaneously associated with both

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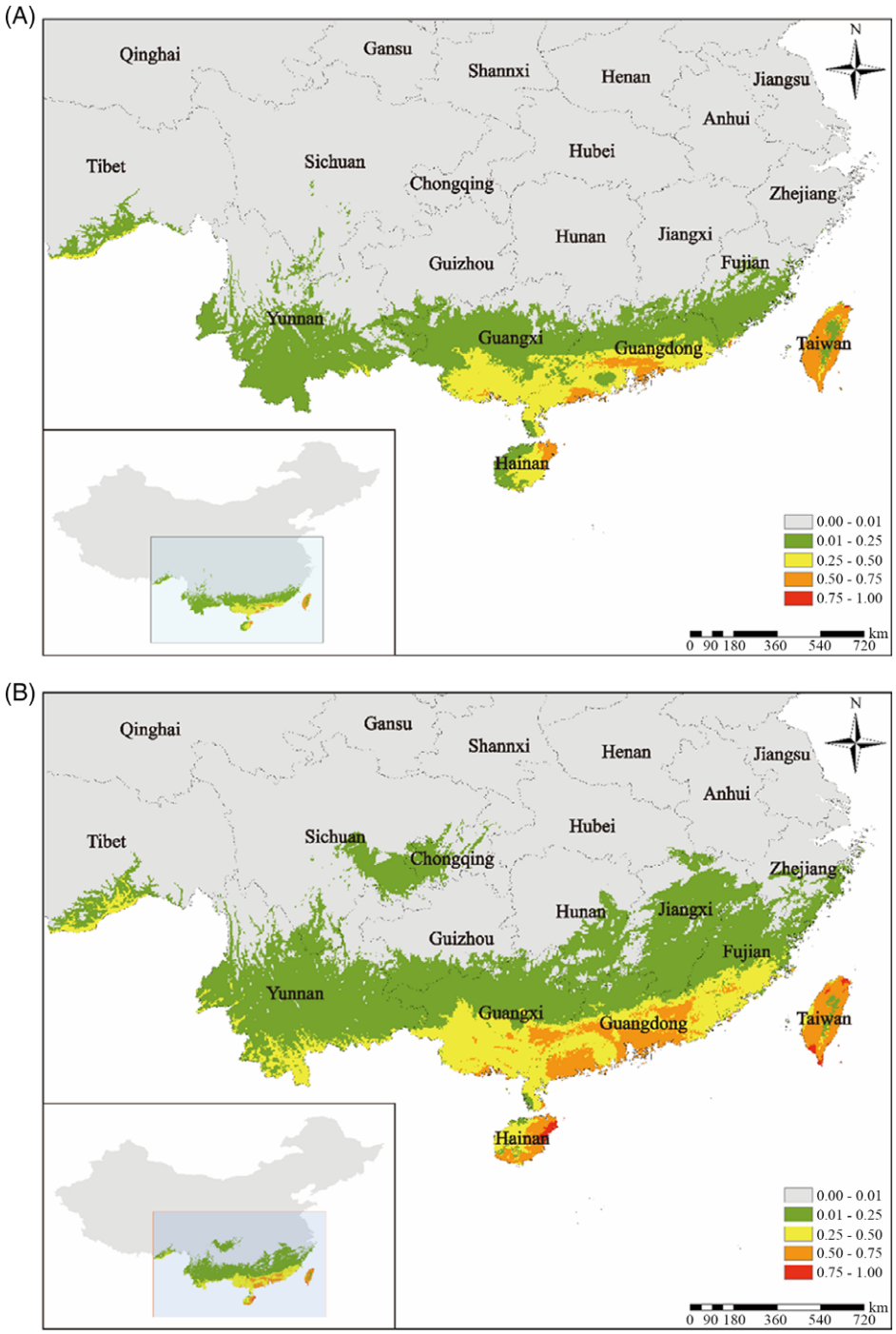

Figure 13. Current (A) and potential future distribution (B) of *Mikania micrantha* predicted based on Maxent.

temperature and precipitation. Similar results have been obtained in other plants like black spruce [*Picea mariana* (Mill.) Britton, Sterns & Poggenb.] (Prunier et al. 2012) and European larch (*Larix decidua* Mill.) (Mosca et al. 2012). These findings suggest that the interaction between temperature and precipitation might be more important than their separate action in causing differentiation at the adaptive (epi)loci. It has been shown that transcriptional networks responsive to dehydration and cold stresses are interconnected in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki 2006).

Mikania micrantha was predicted to expand northward in the future (Figure 13), which is in line with previous suggestion by Hu et al. (2014). Environmental factors, including minimum temperature of coldest month, mean temperature of coldest quarter, mean temperature of driest quarter, temperature annual range, annual precipitation, and precipitation of warmest quarter, made

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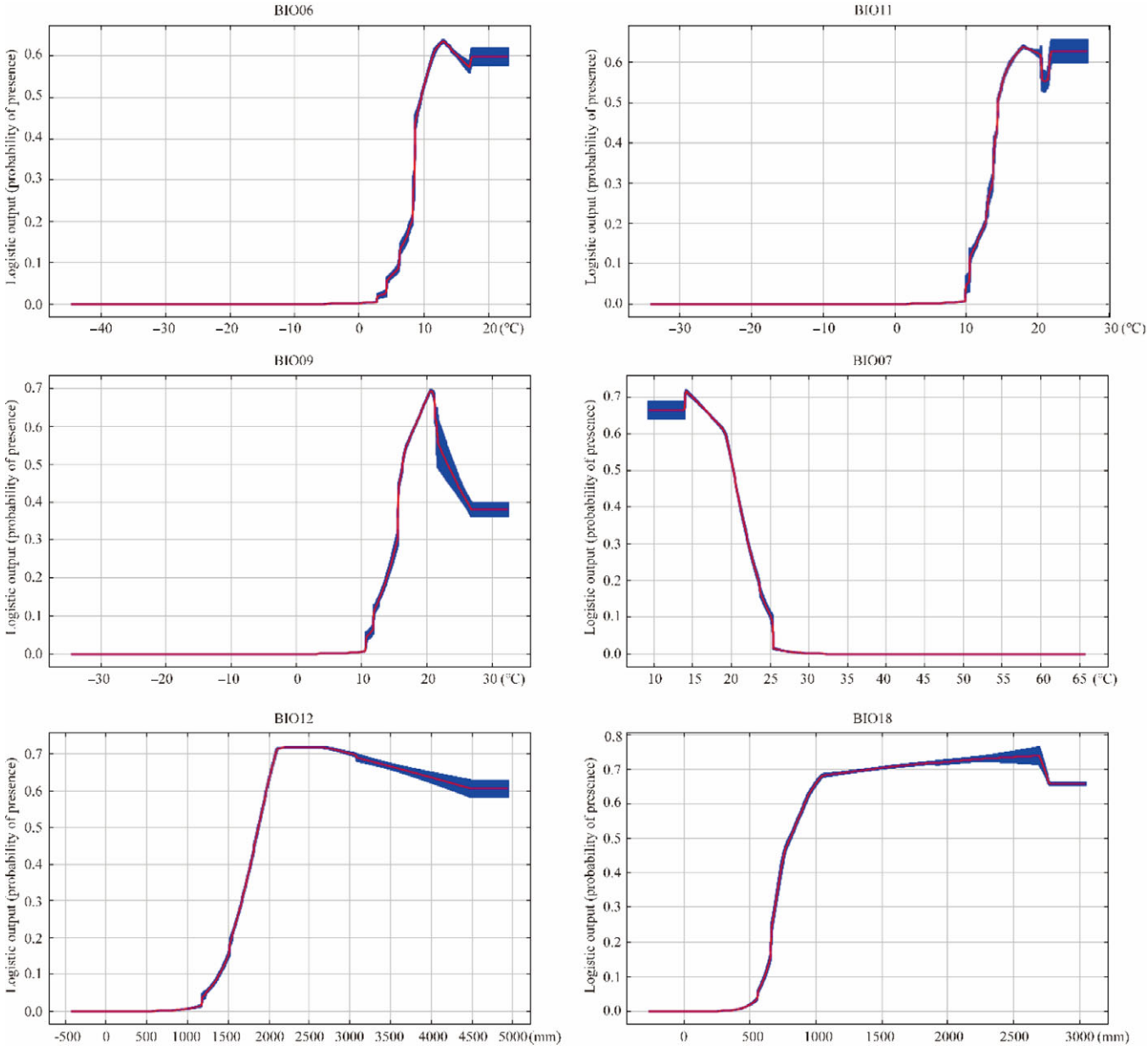

Figure 14. Response curves of *Mikania micrantha* to environmental gradients. The edges of the red and blue curves represent the average response and the standard deviation range, respectively.

the greatest contribution during the expansion (Figure 14). Of them, minimum temperature of the coldest month and mean temperature of the coldest quarter were the decisive factors (contribution rate of 62.3%). More importantly, the optimum growth conditions were revealed as follows (Figure 15): minimum temperature of coldest month, 10 to 24 C; mean temperature of coldest quarter, 14 to 27 C; mean temperature of driest quarter, 16 to 23 C; temperature annual range, 8 to 20 C; annual precipitation, 1,800 to 4,900 mm; and precipitation of warmest quarter, 800 to 3,100 mm.

Soil factors have strong effects on the invasion of *M. micrantha* (Chen et al. 2018). Here, five soil factors (Mn, Zn, Ni, P, and S) were found to be positively related to adaptive (epi)loci. Our results not only highlight the specific selective soil components but also the candidate loci involved in the response to edaphic selection for *M. micrantha*.

Our findings are not unexpected considering the important functions of Mn, Zn, Ni, P, and S. Mn plays a crucial role in many redox reactions as a cofactor for enzymes (Doncheva et al. 2005). Mn deficiency may cause oxidative damage (Shenker et al. 2004), while excess Mn imposes toxic effects by interfering with a plant's use of other mineral elements (Arya and Roy 2011; Doncheva et al. 2005). Zn fulfills functions in auxin synthesis, signal transduction, transcriptional regulation, the defense against reactive oxygen species, and as component of zinc finger protein and enzymes (Bharti et al. 2013; Cakmak 2000; Cherif et al. 2011; Epple et al. 2003;

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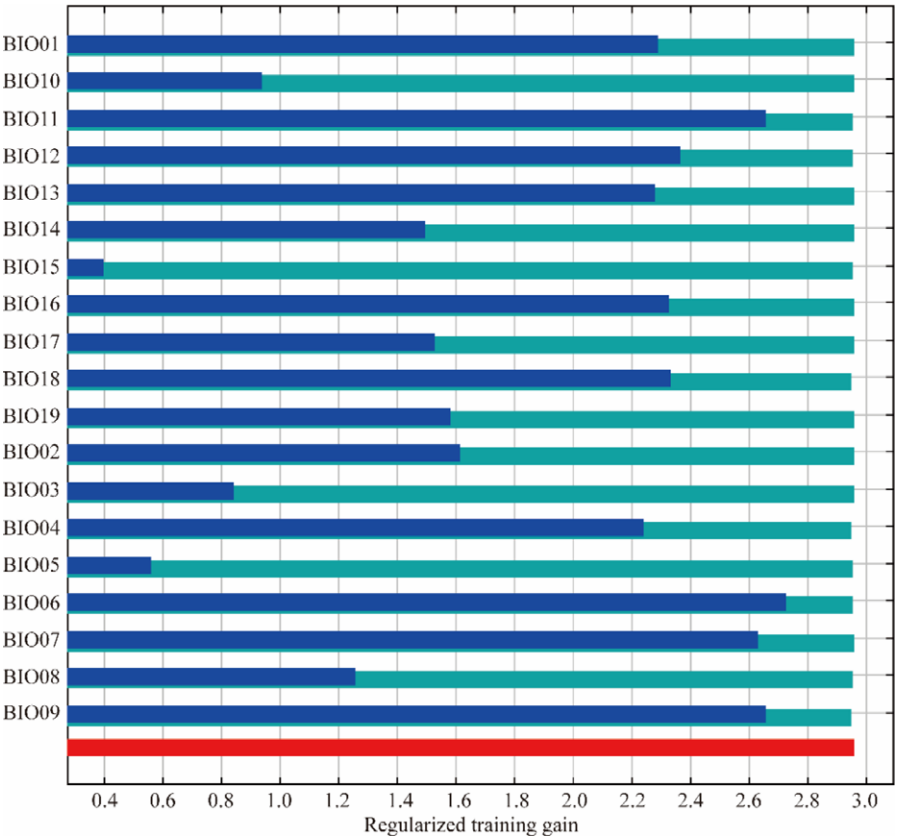

Figure 15. Evaluation of climate variable contribution using the jackknife test. Blue bars represent contribution of each variable; green bars, without variable; red bar, with all variables.

Riechmann et al. 2000). Zn concentration influences germination, branch growth, and light system II (Bonnet et al. 2000). P is a major element in soil organic matter (Herrera-Estrella and López-Arredondo 2016). It participates in many metabolic processes and plays an important role in stress resistance to hostile environments. P excess will affect the absorbance of Fe, Mn, and Zn (Huang 2004). Ni is an important micronutrient for plant growth and metabolism (Lin and Gao 2005). Ni^{2+} generally leads to phytotoxicity in soils (Mishra and Kar1974; Sheoran et al. 1990). Ni may reduce the photosynthetic rate and the activities of key enzymes of the photosynthetic carbon reduction cycle (Sheoran et al. 1990). S represents an essential nonmetallic element for plants (Piotrowska-Dlugosz et al. 2017). Soil S excess and deficiency have serious effects on plant growth (Behera et al. 2020). Of note, S enables increased Ni^{2+} uptake (Hashem et al. 2020). This offers a clue for understanding the preference of *M. micrantha* for heavy metal–contaminated soils (Ho et al. 2019; Leung et al. 2019). This study also provided evidence that selective pressures resulting from soil factors were important in structuring the *M. micrantha* populations (Nosil et al. 2007).

Comparison of Neutral and Adaptive (Epi)Loci Data Sets

Our results showed that adaptive (epi)loci exhibited higher (epi) genetic diversity and differentiation than neutral (epi)loci. Importantly, epigenetic diversity was higher than genetic diversity (Table 8). Compared with neutral loci, adaptive loci data set yielded significantly higher F_{st} values (Table 9). For neutral (epi) loci, the F_{st} value based on the genetic matrix was greater than values based on the epigenetic matrices (Table 9); while the correlation between genetic and geographic distance was weaker than those between epigenetic and geographic distance (Table 10).

By controlling genetic distance, an isolation by geographic distance pattern still can be detected based on the Salmon matrix. After removing adaptive (epi)loci, the Hong Kong and Macao populations were more clearly clustered together based on the genetic and epigenetic matrices (Figure 16), indicating the roles of adaptive (epi)loci in *M. micrantha* invasion. The fine-scale spatial (epi) genetic structure was examined based on neutral and adaptive loci, respectively (Figure 17).

In conclusion, effects of environmental factors on population epigenetic differentiation of *M. micrantha* were first revealed by using MSAP to correlate epigenetic loci and climate/soil data. We found 20 candidate adaptive epiloci correlated with climate (precipitation and temperature) and/or soil variables (Mn, Zn, Ni, P, and S). Minimum temperature of the coldest month and mean temperature of the coldest quarter were identified as decisive factors for *M. micrantha* distribution. Climate is presumed to play a relatively more important role than soil in shaping the adaptive (epi)genetic differentiation. Under ongoing global warming, populations of *M. micrantha* are predicted to expand northward. Compared with genetic diversity, their epigenetic diversity was higher. Population-level (epi)genetic variation showed the pattern

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Table 8. Genetic and epigenetic diversity of neutral and adaptive loci.[a]

		N_a		N_e		I		$\%P$		H_e		UH_e	
Population	N	Neutral	Adaptive	Neutral	Adaptive	Neutral	Adaptive	Neutral	Adaptive	Neutral	Adaptive	Neutral	Adaptive
AFLP													
DG	45	1.040	2.000	1.130	1.818	0.142	0.632	51.8%	100.0%	0.085	0.442	0.086	0.447
HK	91	1.468	2.000	1.102	1.154	0.128	0.214	73.4%	100.0%	0.071	0.117	0.072	0.117
MA	31	0.624	0.909	1.058	1.031	0.072	0.064	31.0%	45.5%	0.041	0.029	0.041	0.029
NLD	62	1.159	2.000	1.122	1.620	0.137	0.507	57.7%	100.0%	0.081	0.347	0.081	0.350
SZ	46	1.073	1.636	1.119	1.482	0.133	0.395	53.5%	81.8%	0.078	0.267	0.079	0.270
ZH	31	0.721	1.818	1.102	1.506	0.108	0.426	35.6%	90.9%	0.066	0.286	0.067	0.290
Average	55	1.014	1.727	1.105	1.435	0.120	0.373	50.5%	86.4%	0.070	0.248	0.071	0.251
Total	306	2.000	2.000	1.114	1.496	0.139	0.488	100.0%	100.0%	0.078	0.315	0.078	0.316
Salmon													
DG	45	1.070	2.000	1.130	1.710	0.145	0.587	53.5%	100.0%	0.086	0.401	0.087	0.406
HK	91	1.570	2.000	1.117	1.137	0.148	0.223	78.5%	100.0%	0.083	0.116	0.083	0.116
MA	31	1.011	1.000	1.103	1.071	0.126	0.123	50.5%	50.0%	0.072	0.062	0.073	0.063
NLD	62	1.248	2.000	1.125	1.836	0.146	0.645	62.4%	100.0%	0.085	0.453	0.086	0.457
SZ	46	1.081	2.000	1.111	1.866	0.131	0.650	54.0%	100.0%	0.076	0.459	0.077	0.464
ZH	31	0.842	2.000	1.105	1.800	0.119	0.632	41.8%	100.0%	0.071	0.441	0.072	0.448
Average	55	1.137	1.833	1.115	1.570	0.136	0.477	56.8%	91.7%	0.079	0.322	0.080	0.326
Total	306	2.000	2.000	1.118	1.731	0.151	0.611	100.0%	100.0%	0.084	0.420	0.084	0.421
H													
DG	45	0.818	2.000	1.065	1.498	0.083	0.374	40.9%	100.0%	0.046	0.258	0.046	0.261
HK	91	1.497	2.000	1.077	1.138	0.110	0.221	74.8%	100.0%	0.058	0.115	0.058	0.116
MA	31	0.971	2.000	1.086	1.356	0.110	0.339	48.5%	100.0%	0.061	0.218	0.062	0.222
NLD	62	1.074	2.000	1.065	1.634	0.089	0.530	53.7%	100.0%	0.048	0.356	0.048	0.359
SZ	46	0.836	2.000	1.053	1.221	0.074	0.305	41.8%	100.0%	0.040	0.172	0.040	0.174
ZH	31	0.662	2.000	1.059	1.924	0.075	0.672	33.0%	100.0%	0.042	0.480	0.043	0.487
Average	55	0.976	2.000	1.067	1.462	0.090	0.407	48.8%	100.0%	0.049	0.266	0.050	0.270
Total	306	2.000	2.000	1.067	1.434	0.102	0.445	100.0%	100.0%	0.052	0.282	0.052	0.282
M													
DG	45	1.081	2.000	1.113	1.821	0.133	0.633	54.1%	100.0%	0.077	0.443	0.078	0.448
HK	91	1.344	1.818	1.077	1.074	0.103	0.137	67.2%	90.9%	0.056	0.066	0.056	0.066
MA	31	0.503	0.727	1.042	1.025	0.054	0.051	25.1%	36.4%	0.030	0.023	0.030	0.023
NLD	62	1.180	1.818	1.107	1.631	0.129	0.508	59.0%	90.9%	0.074	0.351	0.074	0.353
SZ	46	1.043	1.455	1.104	1.391	0.123	0.330	52.1%	72.7%	0.071	0.221	0.072	0.223
ZH	31	0.668	1.818	1.085	1.474	0.095	0.414	33.0%	90.9%	0.057	0.275	0.057	0.279
Average	55	0.970	1.606	1.088	1.403	0.106	0.345	48.4%	80.3%	0.061	0.230	0.061	0.232
Total	306	2.000	2.000	1.092	1.438	0.122	0.447	100.0%	100.0%	0.066	0.284	0.066	0.285
U													
DG	45	0.683	2.000	1.064	1.835	0.076	0.648	34.2%	100.0%	0.044	0.455	0.044	0.460
HK	91	1.409	2.000	1.058	1.422	0.086	0.473	70.4%	100.0%	0.044	0.297	0.044	0.298
MA	31	0.544	2.000	1.037	1.033	0.052	0.083	27.2%	100.0%	0.028	0.032	0.028	0.033
NLD	62	0.921	2.000	1.060	1.016	0.077	0.047	46.0%	100.0%	0.042	0.016	0.043	0.016
SZ	46	0.775	2.000	1.055	1.022	0.069	0.060	38.8%	100.0%	0.038	0.022	0.039	0.022
ZH	31	0.534	0.000	1.054	1.000	0.063	0.000	26.7%	0.0%	0.036	0.000	0.037	0.000
Average	55	0.811	1.667	1.055	1.221	0.071	0.219	40.5%	83.3%	0.039	0.137	0.039	0.138
Total	306	2.000	2.000	1.057	1.223	0.083	0.329	100.0%	100.0%	0.042	0.182	0.042	0.183
HMU													
DG	45	0.862	2.000	1.079	1.807	0.095	0.611	43.1%	100.0%	0.054	0.430	0.055	0.434
HK	91	1.429	1.882	1.072	1.169	0.102	0.220	71.5%	94.1%	0.053	0.122	0.054	0.122
MA	31	0.725	1.059	1.060	1.077	0.079	0.107	36.2%	52.9%	0.044	0.057	0.044	0.058
NLD	62	1.066	2.000	1.076	1.595	0.097	0.493	53.3%	100.0%	0.054	0.334	0.054	0.337
SZ	46	0.882	1.647	1.068	1.399	0.087	0.358	44.1%	82.4%	0.048	0.234	0.049	0.237
ZH	31	0.631	1.882	1.065	1.518	0.078	0.436	31.4%	94.1%	0.045	0.293	0.045	0.297
Average	55	0.933	1.745	1.070	1.427	0.090	0.371	46.6%	87.3%	0.050	0.245	0.050	0.248
Total	306	2.000	2.000	1.072	1.467	0.103	0.462	100.0%	100.0%	0.053	0.296	0.053	0.296

[a]N, number of samples; N_a, number of different alleles; N_e, number of effective alleles = $1/(p^2 + q^2)$; I, Shannon's diversity index = $-1^*[p^*\text{Ln}(p) + q^*\text{Ln}(q)]$; $\%P$, percentage of polymorphic loci; He, expected heterozygosity = 2^*p^*q; UHe, unbiased expected heterozygosity = $[2N/(2N - 1)]^*H_e$; DG, Dongguan; HK, Hong Kong; MA, Macao; NLD, Nei Lingding Island; SZ, Shenzhen; ZH, Zhuhai.

Table 9. Analysis of molecular variance (AMOVA) based on neutral and adaptive loci.

	Neutral loci		Adaptive loci	
Variation sources	Variation percentage	Differentiation value	Variation percentage	Differentiation value
AFLP				
Among populations within groups	9.41%	$F_{sc} = 0.1003$	47.90%	$F_{sc} = 0.0720$
Among populations	9.08%	$F_{st} = 0.1850$	5.89%	$F_{st} = 0.5378$
Among groups	81.50%	$F_{ct} = 0.0941$	46.22%	$F_{ct} = 0.4790$
Salmon				
Among populations within groups	6.53%	$F_{sc} = 0.0720$	53.56%	$F_{sc} = 0.0530$
Among populations	6.73%	$F_{st} = 0.1326$	2.46%	$F_{st} = 0.5602$
Among groups	86.74	$F_{ct} = 0.0653$	43.98%	$F_{ct} = 0.5356$
H				
Among populations within groups	5.22%	$F_{sc} = 0.0733$	32.81%	$F_{sc} = 0.1040$
Among populations	6.95%	$F_{st} = 0.1217$	6.99%	$F_{st} = 0.3980$
Among groups	87.83%	$F_{ct} = 0.0522$	60.20%	$F_{ct} = 0.3281$
M				
Among populations within groups	8.81%	$F_{sc} = 0.0842$	48.88%	$F_{sc} = 0.09377$
Among populations	7.68%	$F_{st} = 0.1649$	4.79%	$F_{st} = 0.53670$
Among groups	83.51%	$F_{ct} = 0.0881$	46.33%	$F_{ct} = 0.48877$
U				
Among populations within groups	6.58%	$F_{sc} = 0.0883$	26.60%	$F_{sc} = 0.2459$
Among populations	8.22%	$F_{st} = 0.1480$	18.05%	$F_{st} = 0.4465$
Among groups	85.20%	$F_{ct} = 0.0658$	55.35%	$F_{ct} = 0.2660$
HMU				
Among populations within groups	6.66%	$F_{sc} = 0.0799$	42.29%	$F_{sc} = 0.1054$
Among populations	7.45%	$F_{st} = 0.1411$	6.08%	$F_{st} = 0.4837$
Among groups	85.89%	$F_{ct} = 0.0666$	51.63%	$F_{ct} = 0.4229$

Table 10. Mantel test based on neutral and adaptive locus matrices.

	Neutral loci		Adaptive loci	
Matrix	r^2	P	r^2	P
AFLP	0.1690	0.0255	0.2408	0.0094
Salmon	0.2327	0.0044	0.2676	0.0056
H	0.1973	0.0202	0.2460	0.0159
M	0.1443	0.0469	0.2382	0.0100
U	0.1178	0.0962	0.0003	0.4452
HMU	0.1958	0.0124	0.2563	0.0073

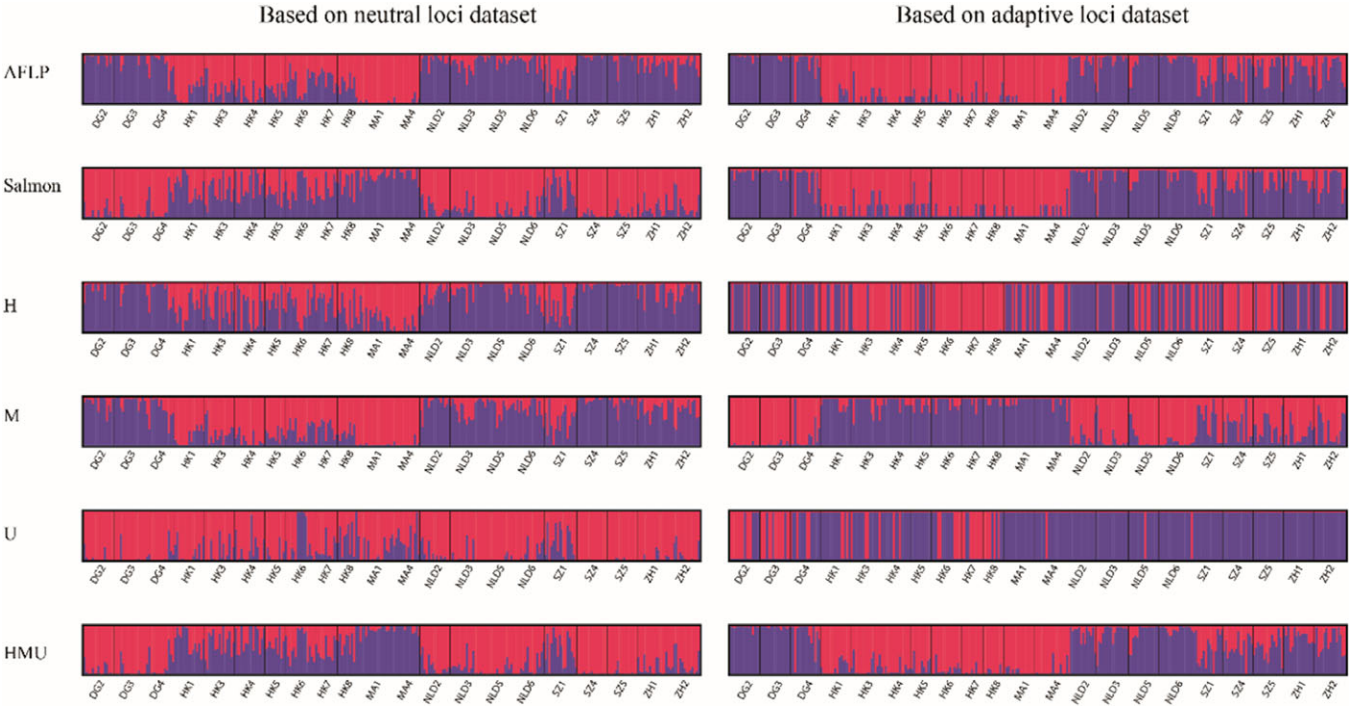

Figure 16. Structure results of neutral or adaptive loci based on the six matrices. See Table 1 for population codes.

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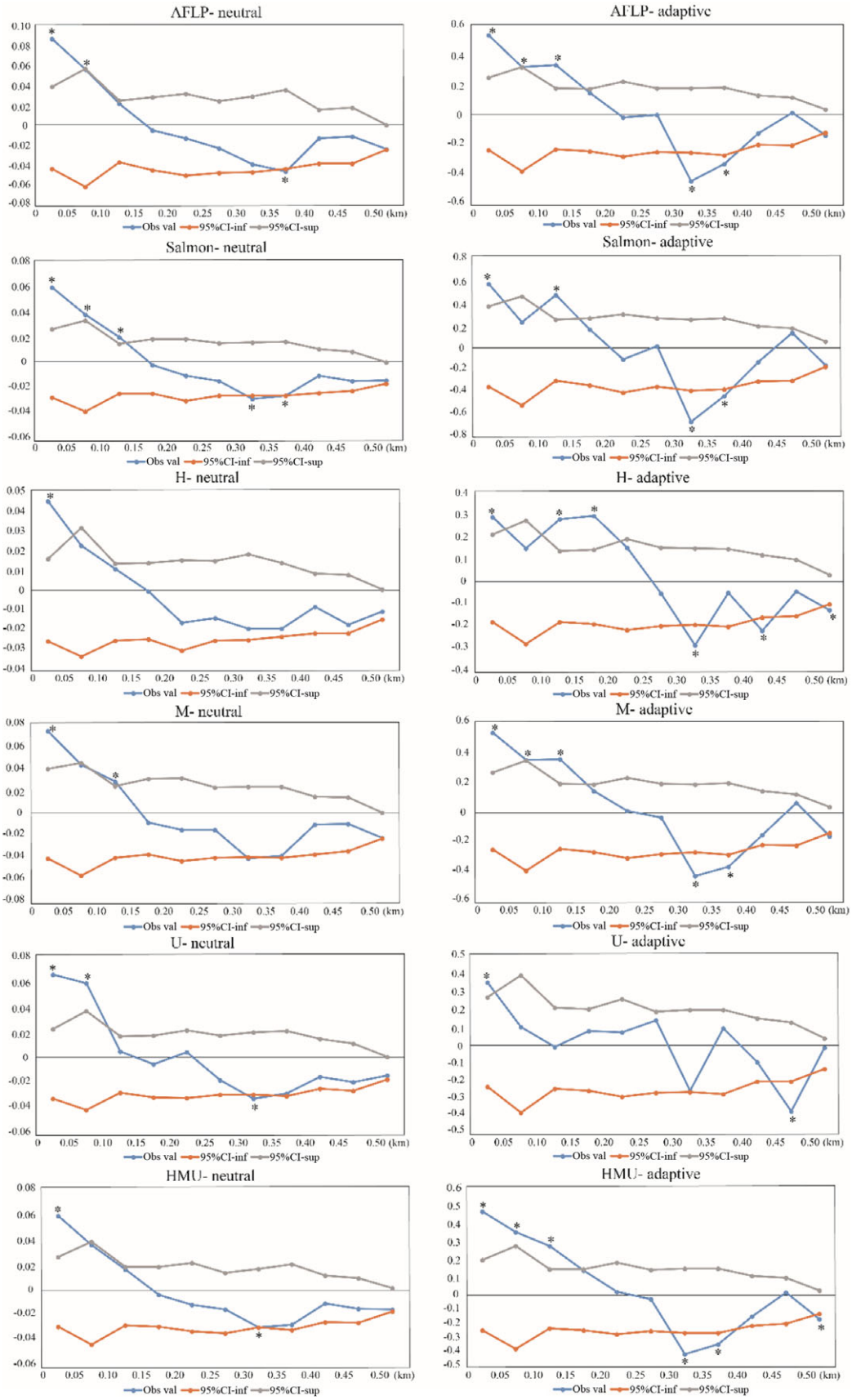

Figure 17. Fine-scale spatial structure of neutral and adaptive loci based on the six matrices. An asterisk (*) indicates significance level ($P < 0.05$).

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of isolation by distance and spatial structure at a small scale. Moreover, leaf shape variation was found to be related to population methylation percentage and epigenetic diversity. These results may be helpful for formulating a control strategy for *M. micrantha*.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2021.13

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