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ORIGINAL PAPER

On the origin and genetic variability of the two invasive biotypes of *Chromolaena odorata*

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Abstract *Chromolaena odorata* (L.) R. M. King and H. Robinson (Asteraceae), originally from the Neotropics, has become a serious weed in the humid tropics and subtropics of Southeast Asia, Africa and Pacific Islands. In its introduced distributions, *C. odorata* has been recognised as two biotypes, the Asian/West African (AWA) biotype and South African (SA) biotype, with independent distribution, morphology and ecological characters. To characterise the genetic variability and identify the likely source regions in the native distributions of the two biotypes, we carried out an extensive phylogeographic study using chloroplast and nuclear DNA sequences and microsatellite DNA markers. The analysis of both DNA sequences and nuclear markers showed that native populations possessed high genetic diversity, while both the AWA and SA biotypes in invaded regions appeared to have low genetic diversity. The AWA and SA biotypes were genetically distinct. Strong competitive ability and environmental adaptability may have facilitated the invasion AWA and SA biotypes in its respective invasive regions. We conclude that the source of AWA biotype may be Trinidad and Tobago, while the SA biotype was from Cuba and Jamaica. For a better outcome of biocontrol, the potential biological control agents for the two biotypes should be collected from these native regions, respectively.

Keywords *Chromolaena odorata* · Invasion · Biotype · Genetic diversity · Source location · Biological control

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Introduction

Chromolaena odorata (L.) King and Robinson (Asteraceae) is a perennial shrub native to Central and South America, from Florida to northern Argentina (Gautier 1992). It has become an invasive weed in the humid

tropics and subtropics of Southeast Asia, Africa and Pacific Islands (Gautier 1992; Ye et al. 2004). In its introduced ranges, *C. odorata* grows rapidly and often forms a dense thicket scrambling and smothering other local plants, causing significant damages to biodiversity, agriculture, forestry and ecotourism (Goodall and Erasmus 1996; Norbu 2004; Zachariades et al. 2009).

In the introduced habitats, *C. odorata* was observed to exhibit two biotypes, i.e. Asian/West African (AWA) biotype and the southern African (SA) biotype (Zachariades 2003; Codilla and Metillo 2012). The AWA biotype was believed to be introduced to Serampore Botanical Garden in Calcutta, India as an ornamental plant in the 1840s. Infestations were then thought to have arisen throughout Southeast Asia and parts of Oceania (McFadyen 1989, 2002). In the late 1930s, the AWA biotype of *C. odorata* was introduced into Nigeria from Sri Lanka through the unintentional importation of contaminated seed of *Gmelina arborea* (Ivens 1974). Following this introduction, it has gradually spread to most regions of the West Africa and eventually reached the Central and East Africa (Muniappan et al. 2005; Zachariades et al. 2009; Uyi and Igbinosa 2013). While plants identical to the AWA biotype exist widely in its native distributions (Zachariades et al. 2009), its exact origin of remains unclear.

McGibbon (1858) recorded that *C. odorata* in southern Africa (SA biotype) was first introduced from Jamaica to a botanical garden in Cape Town, while other literature suggests that *C. odorata* was first introduced into South Africa accidentally as seed-contaminated packing material off-loaded at Durban harbour during or before the 1940s (Pickworth 1976; Zachariades 2003). Plants from the Northern Caribbean region, such as Cuba, Jamaica and Puerto Rico, at a macro-scale, were similar to the SA biotype, but none matched it in the entirety of characters (Kluge 1991; Zachariades et al. 1999; Paterson and Zachariades 2013), which made it difficult to confirm the source of this biotype. It is worth mentioning that a single specimen which appears identical to AWA biotype was found in Zimbabwe in the late 1960s (Gautier 1992). If this could be linked to an unconfirmed *C. odorata* infestation in northern Mozambique and southern Malawi, Mozambique would be the only country with both AWA and SA biotypes of *C. odorata* (Zachariades et al. 2009). This sample, however, was not included in any genetic study.

Phylogeographic analyses of intraspecific genetic variation could characterise the genetic diversity among populations and address the origins of invasive species (e.g., Schaal et al. 2003, 2010; Perdereau et al. 2013). By analysing DNA sequence data of the internal transcribed spacer 1 (ITS-1) of 11 samples from Thailand, Indonesia, north Queensland, Ivory Coast, South Africa, Brazil, Colombia, and the United States, Scott et al. (1998) demonstrated that the most common form of *C. odorata* in Australia was genetically similar to the Asian form, and that Brazil was one of the most likely sources of the rare form in Australia. von Senger et al. (2002) and Barker et al. (2005) both showed that samples from South Africa have more than one genotype, while they identified no correlation between intraspecific genetic variation and morphological diversity of *C. odorata* from the native and introduced range. Using inter-simple sequence repeats (ISSRs), Paterson and Zachariades (2013) strongly supported the distinct genetics between AWA and SA biotypes and identified Jamaica and Cuba as the possible origin of SA biotype, and Florida, Venezuela and Trinidad as the possible origin of AWA Biotype. However, Paterson and Zachariades (2013) only had four samples of AWA biotype which was probably not sufficient to represent the vast distribution of this biotype across the tropical Asia, Oceania and West Africa. Moreover, it was impossible to distinguish homozygosity and heterozygosity through ISSR markers (Feng et al. 2016). In the phylogeographic analysis, multiple sequences and various molecular methods are needed to obtain the full potential of phylogeographical analysis (Schaal et al. 2010). Based on DNA sequencing (cpDNA and rDNA) and microsatellite DNA (also known as simple sequence repeat, SSR), Yu et al. (2014) suggested that the most likely origin of Asian *C. odorata* was Trinidad and Tobago or adjacent areas in the West Indies. However, no samples were collected from Africa, Western Pacific islands and Oceania in Yu et al. (2014). Therefore, it would be of particular interest to validate conclusions from previous studies (e.g., Paterson and Zachariades 2013; Yu et al. 2014) through using more informative genetic markers and investigating sufficient number of samples from its global distribution.

Despite different attributes between the two biotypes, surprisingly little is known about their genetic variability and relatedness. The study of genetic

diversity within populations of invasive weed may provide important information in understanding the mechanisms of invasion success (Perdereau et al. 2013). In some cases, high genetic diversity could increase the capacity of invaders to be adapted in novel environments, thus promoting successful invasion (Wang et al. 2012; Forsman 2014). However, low genetic diversity within invasive populations was also revealed in many species. Therefore, successful invaders may not necessarily have high genetic diversity (Meimberg et al. 2005; Geng et al. 2007; Dlugosch and Parker 2008; Perdereau et al. 2013).

Insect-plant incompatibility was regarded as one of the most likely factor causing the failure of biocontrol (Zachariades et al. 2009). Information of genetic origin of *C. odorata* population is essential for the source of locally adapted biological control agent populations. In this study, we collected samples of *C. odorata* from its global distribution, and employed multiple molecular markers, with aims to (1) characterise the genetic variability and genetic structure of native and invasive populations of *C. odorata*; (2) identify the geographical origin of the two biotypes of *C. odorata*. To this end, we built a genetic dataset by sequencing 140 samples (with additional 247 samples from our previous study) for two cpDNAs (*psb*A-*trn*H and *atp*B-*rbc*L), one rDNA (ITS5p-ITS8p), and by genotyping six microsatellite loci. The results from this study could provide the basis for the management of this invasive plant species.

Materials and methods

Material collections and DNA extractions

A total of 92 individuals of *C. odorata* from native (Nat) distributions were sampled from 14 countries broadly spanning the native range. Forty-eight samples were collected from the invaded region: twenty-one from Asia, six from West Pacific Islands and Oceania (WPIO), two from West Africa (WA), and nineteen from South Africa (SA). We presented the information on each sampling location and the details of population division in Supplementary 1, 2. Leaves of *C. odorata* were collected and dried using activated silica gel. Total genomic DNAs were extracted from the dried leaves using the modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1987). The remaining leaf materials and DNAs were stored at the Lab of Community Assembly and Species Coexistence Group in Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences. All the 140 individuals were used for DNA sequencing, while 36 of 48 individuals from invaded ranges were used for the microsatellite analysis (DNA of other samples were substandard as the low consistency).

DNA sequencing and microsatellite DNA genotyping

We used a specific primer of *C. odorata* for cpDNA intergenic spacers *atp*B-*rbc*L1, a universal plastid primer for *psb*A-*trn*H (Sang et al. 1997; Tate 2002), and a universal nuclear primer for ITS (Möller and Cronk 1997) (Table 1). Except for the annealing temperature that was specified in Table 1, the amplification protocols followed Yu et al. (2014). PCR products were sequenced using an ABI 3730 DNA Sequence Analyser (Applied Biosystems, Foster City, California, USA). All generated sequences were deposited in GenBank (accession numbers from KY985820 to KY986239). The SSR markers and PCR amplification procedures were taken from Yu et al. (2014) for genotyping the 36 individuals from invasive ranges.

Analysis of genetic diversity and haplotype network

The cpDNA and ITS sequences were edited and assembled using Geneious 4.8.5 (Kearse et al. 2012). Additional DNA sequences used in Yu et al. (2014), including ITS and cpDNA sequences for 119 samples across the native region of *C. odorata* and 129 samples from ten Asian countries, were also obtained from Genbank, and combined with new data for analysis (Suppl. 3). We manually aligned and subsequently adjusted multiple DNA sequences using Mega 7.0 (Kumar et al. 2016). We combined the two cpDNA regions for the downstream analyses. For the nuclear genes, we used standard nucleotide ambiguity codes to identify heterozygous sites with double peaks in chromatograms and resolved the nuclear sequences using PHASE (Stephens et al. 2001; Stephens and Donnelly 2003) in software package DNAsp v 5.0 (Librado and Rozas 2009).

Table 1 Primers of selected molecular markers used in sequencing and microsatellite analysis of *Chromolaena odorata*

Locus name	Primer sequence (5′–3′)	Ta (°C)	Repeats	Source
atpB-rbcL1	atpB: CGAATACGGGACCAATAATTTGGGCGA rbcL: ACCAGCTTTGAATCCAACACTTGCTT	60	–	Designed using Geneious 4.8.5 (Kearse et al. 2012)
psbA-trnH	psbA: GTTATGCATGAACGTAATGCTC trnH2: CGCGCATGGTGGATTCACAATCC	55	–	Sang et al. (1997), Tate (2002)
nrITS	ITS5p: GGAAGGAGAAGTCGTAACAAGG ITS8p: CACGCTTCTCCAGACTACA	58	–	Möller and Cronk (1997)
AC26	F: CAGACTGGATCATAAGAA R: TTACGTGTAATAGAGCCT	58	(TG)8 … (TG)3	Yu and Li (2011), Yu et al. (2014)
AC50	F: TACCCTGTTATTCCCACT R: CCTAAGCCTTCTTATTTGAT	60	(TG)10	Yu and Li (2011), Yu et al. (2014)
AG65	F: CAGTTATCTTCAACACCCAA R: TTTCCGACTAAACCCATC	58	(CT)7 … (CT)4 …(TC)3	Yu and Li (2011), Yu et al. (2014)
AG115	F: TCGTGGTAGAGCAGAAGA R: AACTGCCAGATCAGGTTG	54	(AG)6 GTT(AG)4	Yu and Li (2011), Yu et al. (2014)
AG189	F: AGAGTAAGCACGAGACCG R: AGAACTTTACCTCCCACA	60	(TTTTG)3 … (AG)9	Yu and Li (2011), Yu et al. (2014)
AG227	F: GTTCGTCACCCTTTTCTC R: ATCTGCACTTCATCTTCTTC	62	(GA)5 … (AG)9	Yu and Li (2011), Yu et al. (2014)

F forward primer; *R* reverse primer; *Ta* annealing temperature

The recombination in nuclear genes (Rm) and total haplotype diversity (H_D), nucleotide diversity (π) of cpDNA and ITS sequences were calculated using DNAsp v5.10 (Librado and Rozas 2009). We categorised the samples into five groups: Nat, Asia, WPIO, WA (two samples in total) and SA, and carried out analysis on H_D, π and the genetic distance (F_{ST}) between different groups in DNAsp v5.10 (Librado and Rozas 2009). Because we revealed a high consistency of haplotype in Asia, West Pacific Islands, Oceania and West Africa (AWA biotype), and extremely low haplotype categories in South Africa (SA biotype) (see "Results" section), we further pooled the samples into AWA group and SA group for the remaining analyses.

The degree of relatedness among cpDNA and ITS haplotypes was estimated using Network v 5.0 following a Median-Joining method (Bandelt et al. 1999). We chose *Ageratina adenophora* (Spreng.) R. M. King and H. Rob. as the outgroup since *Ageratina* is thought to be basal to *Chromolaena* (Schmidt and Schilling 2000). According to the global position coordinates of the sampling sites, a geographic distribution of the haplotypes of *C. odorata* was generated using the software ArcGIS version 10.2 (http://desktop.arcgis.com). We also recombined all the DNA sequences (cpDNA-ITS) and mapped the haplotypes distribution of invasive individuals and the distribution of these haplotypes in native regions to identify the native individuals that shared the same haplotype distributions with samples from SA or AWA group in both cpDNA and ITS sequences.

Microsatellite DNA data were edited and formatted in GenAlEx v6.4 (Peakall and Smouse 2006). Input files with an appropriate format for other software analysis were also created in GenAlEx. Because populations of the AWA group had extremely low genetic differentiation, we pooled them into one group (population) for further analyses. Genetic diversity indices, including observed heterozygosity (H_O) and expected heterozygosity (H_E) were calculated using POPGENE v1.32 (Yeh et al. 1997). The allelic number (A), and richness (A_R) were estimated in FSTAT v2.9.3 (Goudet 2001). The index for each population from South Africa and the native distributions are also calculated. Tests for departure from Hardy–Weinberg

equilibrium (HWE) were performed using POPGENE v 1.32 (Yeh et al. 1997).

We further combined alleles at all six microsatellite loci as multi-locus genotypes (MLGs) to be consistent with the comparable research (Yu et al. 2014), and the SSR data from Yu et al. (2014) were also combined with the new data for further analysis. The genotypic frequency was estimated in ARLEQUIN v 3.0 (Excoffier et al. 2007). To estimate the genetic variation within and among populations of the three groups, an analysis of molecular variance (AMOVA) was implemented for both DNA sequences and microsatellite loci in ARLEQUIN v 3.0 (Excoffier et al. 2007), and the significance was tested using 10^4 permutations (Excoffier et al. 1992). Population genetic structure was assessed with Wright's fixation index (Wright 1950). We also calculated the global F_{ST} (Weir and Cockerham 1984) for populations in the three groups.

Population genetic structure was further tested using a Bayesian clustering analysis in STRUCTURE v 2.2.3 (Pritchard et al. 2000) using SSR data. The simulation was run with values of expected clusters (K) from 1 to 20 and was repeated 20 times for each set. For each run, an admixture (the default value) and a correlated-allele frequencies model was combined with a burn-in of 10^5 iterations and 10^5 subsequent MCMC steps (Pritchard et al. 2000). The optimal value for groupings was evaluated using ΔK based on the log-likelihood value (Excoffier et al. 2007). Finally, genetic distances matrix among microsatellite phenotypes were calculated in MSAnalyser (Dieringer and Schlötterer 2003), and the pop-based principal coordinate analysis (PCoA) was visualised with MVSP, version 3.1 (Kovach 1999).

Results

Genetic structure as revealed by cpDNA and ITS

A total of 387 samples with cpDNA and ITS sequences was analysed, with 140 newly sequenced, and 247 samples from Yu et al. (2014). The length of the two combined cpDNA fragments in *C. odorata* was 1420 bp, with 552 bp of *psb*A-*trn*H fragment and 868 bp of *atp*B-*rbc*L, respectively. The length of the ITS fragment was 659 bp. The ITS sequences of the 136 individuals (61.5%) in native regions showed heterozygous sites that were also found in almost all individuals (except MpL4) from South Africa. Including gaps, there were 71 and 140 polymorphic sites in cpDNA and ITS sequence datasets, resulting in 16 cpDNA and 38 ITS haplotypes. The AMOVA results revealed that there was more variation (48.3%) partitioned among populations rather than among groups (27.0%) and within (24.6%) populations based on cpDNA data. Less variation (29.2%) was partitioned among populations than variation among groups (35.7%) and within populations (35.7%) based on the nuclear gene. Significant genetic differentiation was also revealed among the populations and groups (Table 2).

For both cpDNA and ITS fragments, Nat group contained the most haplotypes (cpDNA, $h = 15$; ITS, $h = 37$), the highest level of haplotype diversity (cpDNA, $H_D = 0.7875$; ITS, $H_D = 0.7836$), and high nucleotide diversity (cpDNA, $\pi = 0.0016$; ITS, $\pi = 0.0104$). The Asia, WPIO and WA groups shared the same cpDNA and ITS haplotype, and genetic distances between the three groups were zero. We therefore combined samples from the three groups and renamed the groups as "AWA". The SA group also only had one haplotype in the cpDNA sequence ($h = 1$, $H_D = 0$, $\pi = 0$), while two haplotypes in the ITS sequence ($h = 2$, $H_D = 0.51209$, $\pi = 0.00789$). The SA group had closer genetic distance with Nat group (cpDNA, $F_{ST} = 0.2645$; ITS, $F_{ST} = 0.0419$) than with AWA group (cpDNA, $F_{ST} = 1.0000$; ITS, $F_{ST} = 0.5135$). The genetic distance between AWA and Nat group was intermediate (cpDNA, $F_{ST} = 0.50377$; ITS, $F_{ST} = 0.43971$). The minimum number of recombination events detected from ITS sequences is 11 (Rm = 11).

The phylogeographic network analysis showed that both the cpDNA and ITS haplotype of AWA group were the same as the dominant ones in Asia (cpDNA h1 and ITS h1), as reported in Yu et al. (2014). Our results showed that, besides the native countries and regions found in Yu et al. (2014), i.e. Florida, Trinidad and Tobago, this single cpDNA haplotype (h1) were also found in many other native countries, i.e. Jamaica, Puerto Rico, Venezuela, Guyana, Paraguay (Figs. 1, 2); Besides Florida, Trinidad and Tobago, Puerto Rico, Jamaica and Venezuela found in Yu et al. (2014), Costa Rica, Cuba, Dominican Republic, Guyana and Paraguay also shared the single ITS haplotype (h1) (Figs. 1, 2). All samples from AWA

Table 2 Results of the analysis of molecular variance (AMOVA) for cpDNA, ITS and SSR loci of *Chromolaena odorata*

Locus	Source of variation	*df*	SS	VC	Variation (%)	Fixation indices
cpDNA	Among groups	2	621.611	1.70584	26.99	$F_{CT} = 0.26992$*
	Among populations within groups	38	800.487	3.05709	48.37	$F_{SC} = 0.66259$*
	Within populations	345	537.086	1.55677	24.63	$F_{ST} = 0.75366$*
	Total	385	1959.184	6.3197		
ITS	Among groups	2	492.693	1.15801	35.66	$F_{CT} = 0.97961$*
	Among populations within groups	72	778.677	0.94792	29.19	$F_{SC} = 0.64851$*
	Within populations	695	793.269	1.14139	35.15	$F_{ST} = 0.45370$*
	Total	769	2064.639	3.24733		
SSR loci	Among groups	2	318.179	0.64845	39.72	$F_{CT} = 0.39719$*
	Among populations within groups	55	332.131	0.27669	16.95	$F_{SC} = 0.28115$*
	Within populations	1058	748.492	0.70746	43.33	$F_{ST} = 0.56667$*
	Total	1115	1398.802	1.6326		

Populations were categorised into three groups (AWA, SA and NAT groups)

df degree of freedom, *SS* sum of squares, *VC* variance components, F_{CT} genetic differentiation among groups, F_{SC} genetic differentiation among populations within groups, F_{ST} genetic differentiation index

Level of significance: $*P < 0.01$

group had a single haplotype (hap-AWA) of cpDNA-ITS sequences and this single haplotype also appeared in native counties and regions including Florida, Jamaica, Puerto Rico, Venezuela, Trinidad and Tobago, Guyana and Paraguay (Fig. 1).

The SA group had a single cpDNA haplotype (h2) and two ITS haplotypes (h1 and h2), and all the three haplotypes were widely distributed across the native countries (Figs. 1, 2). While in five native regions (Cuba, Jamaica, Dominican Republic, Puerto Rico, Venezuela), ITS haplotypes h1 and h2 both exist, which shared the same haplotypes distribution with SA group (Figs. 1, 2). Different from all the other individuals in SA group in our study, one individual (MpL4) in SA group lacked the heterozygous sites in ITS sequence with a single haplotype h2 that were also found in one individual from South Africa in Barker et al. (2005) (AY576817) and two individuals from Cuba (Cub4 and Cub5) in this study. Based on the above results, we define hap-SA1 as individuals that had the same two haplotypes of cpDNA-ITS sequences, and hap-SA2 as individuals that had a single haplotype. The result showed that hap-SA1 was found in Jamaica, Cuba, Dominican Republic, Puerto Rico and Venezuela, while hap-SA2 was only in Cuba (Fig. 1).

Genetic structure revealed from microsatellite DNA (SSR)

A total of 558 samples were included in the SSR analysis, including 36 newly genotyped, and 522 samples from Yu et al. (2014). The observed heterozygosity (H_O) of all six SSR loci in the AWA group (548 samples) and four loci (AG65, AG115, AG189 and AG227, except the AC26 in MpL population, AG115 in KZZ population) in the SA group (10 samples) were either 0 or 1 (Table 3), indicating a high concordance of the genotypes in these loci. In the Nat group, the number of alleles of the six SSR loci (6–10) was significantly higher than that in the two invasive groups (Table 3). The native group had the highest allelic richness (A_R) (Table 3), and populations from Mexico had the highest A_R (Suppl. 4). Few populations had positive fixation indices (F), indicating a deficiency of heterozygotes and significant deviations from HWE at multiple loci and at a few populations ($P < 0.01$) (Suppl. 4). The AMOVA revealed that the majority of genetic variation existing within populations (43.33%) and among groups (39.72%), and the significant genetic differentiation coefficient $F_{ST} = 0.167$ and $F_{CT} = 0.397$ (Table 2).

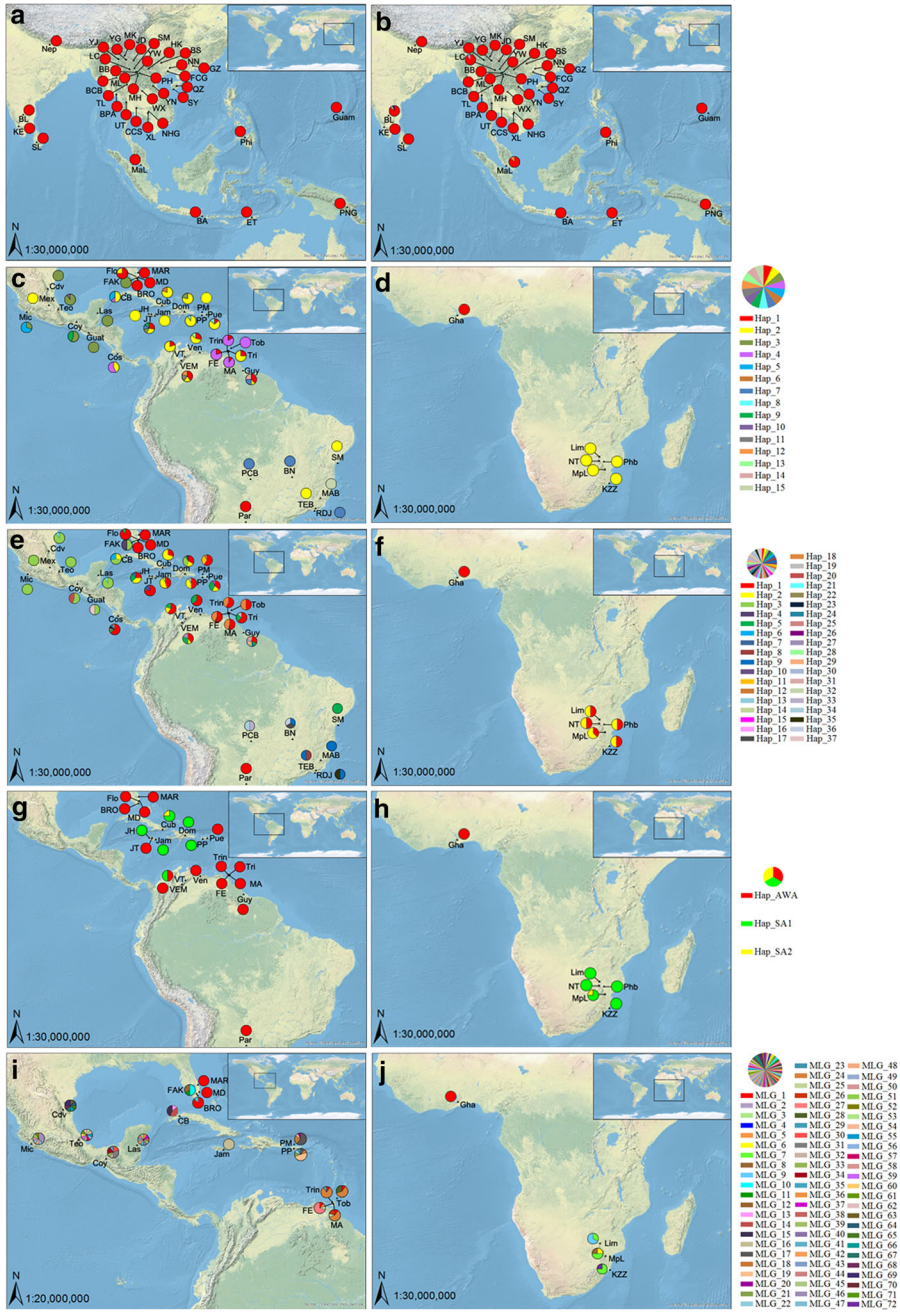

Fig. 1 The distribution of haplotype in *Chromolaena odorata*. **a** Distribution of haplotypes of cpDNA, ITS and cpDNA-ITS in Asia, Western Pacific Islands and Oceania. **b** Frequency of MLGs in Asia, Western Pacific Islands and Oceania. **c** Distribution of cpDNA haplotypes in native regions. **d** Distribution of cpDNA haplotypes in African regions. **e** Distribution of ITS haplotypes in native regions. **f** Distribution of ITS haplotypes in African regions. **g** Distribution of cpDNA-ITS haplotypes in native regions. **h** Distribution of cpDNA-ITS haplotypes in African regions. **i** Frequency of MLGs in native regions. **j** Frequency of MLGs in African regions. Population codes refer to Suppl. 2. Maps were drawn using the software ArcGIS version 10.2 (http://desktop.arcgis.com) and modified using Photoshop (Adobe Corporation, California, America)

In total, 72 MLGs were found in the 558 individuals. All the nine newly genotyped populations in AWA group had only a single MLG that was the same as the predominant one as previous researched in Yu et al. (2014) (Fig. 1). This MLG was also found in three populations in Florida and all four populations in Trinidad and Tobago (Fig. 1). In the SA group, five MLGs were not found in any other populations (Fig. 1). The principal coordinate analysis (PCoA) revealed three general groups, including populations of AWA, BRO, MAR, MD, Trin, Tob, FE and MA in one group (Clade I), populations of MpL, Lim, KZZ, CB, Jam, PP and PM in the second group (Clade II), while the remaining individuals all from native populations as the third group (Clade III) (Fig. 3).

Table 3 Genetic diversity for AWA, SA and native groups of *Chromolaena odorata* based on six SSR loci

Locus	AWA					SA					NAT				
	N	H_O	H_E	*A*	A_R	*N*	H_O	H_E	*A*	A_R	*N*	H_O	H_E	*A*	A_R
AC26	382	0.000	0.000	1	1.0	4	0.250	0.536	2	2.0	153	0.209	0.702	6	3.4
AC50	386	1.000	0.501	2	2.0	10	0.800	0.505	2	2.0	155	0.561	0.822	10	4.7
AG65	386	0.003	0.005	3	1.0	10	0.000	0.000	1	1.0	143	0.462	0.791	11	4.4
AG115	389	0.000	0.000	1	1.0	10	0.100	0.100	2	1.4	154	0.104	0.580	7	2.9
AG189	393	0.005	0.008	3	1.0	10	1.000	0.526	2	2.0	154	0.649	0.868	15	5.3
AG227	392	1.000	0.503	4	2.0	10	1.000	0.526	2	2.0	153	0.536	0.766	16	4.6
Mean	388.000	0.335	0.169	2.333	1.343	9.000	0.525	0.366	1.833	1.732	152.000	0.420	0.755	10.833	4.206
SE	1.693	0.210	0.105	0.494	0.209	1.000	0.188	0.101	0.167	0.176	1.826	0.088	0.042	1.662	0.365

N number of samples, H_O observed heterozygosity, H_E expected heterozygosity, *A* number of alleles, A_R allelic richness, *SE* standard error

Bayesian population structure analysis showed that $K = 2$ was the optimal *K* value, which separated the 58 populations of *C. odorata* into two distinct clusters (Fig. 4). One cluster contained the majority individuals from AWA group and 23 individuals from three populations in Florida, all four populations from Trinidad and Tobago, and one population from Mexico. All the three populations from SA group and the remaining native populations formed the other cluster. The result of $K = 10$ was presented to illustrate the further subdivision among the populations (Fig. 4). When $K = 10$, the dominant MLG were shared between the same populations as those in $K = 2$, while the populations from SA group, including MpL, Lim and KZZ, shared same MLGs with Jamaica and Cuba.

Discussion

In this study, we employed both chloroplast and nuclear DNA sequences and SSR analyses on *C. odorata* samples collected from its global distribution including both native and invasive regions. The results confirmed that the SA biotype and the AWA biotype are genetically distinct. As recorded in previous literature (e.g., Mcfadyen and Skarratt 1996; McFadyen 1989; Zachariades et al. 2009) and verified in several genetic studies (Yu et al. 2014; Paterson and Zachariades 2013), the two biotypes indeed had different origins. Agents for future biological control of AWA biotype should mainly consider from Trinidad and Tobago, and SA biotypes should from Cuba and Jamaica.

Genetic diversity and successful invasion of *C. odorata*

Paterson and Zachariades (2013) showed that the AWA and SA biotypes were genetically distinct. Our results, based on extensive samples coverage and various analyses confirmed the presences of two distinguishable genotypes of *C. odorata* in its invaded ranges. In AWA biotype populations, a single haplotype of cpDNA and ITS, and one dominate MLGs confirmed the high level of genetic consistency of this biotype. Four of the six SSR loci in SA group exhibited a high uniformity and revealed a very low genetic diversity of SA biotype. Our study revealed

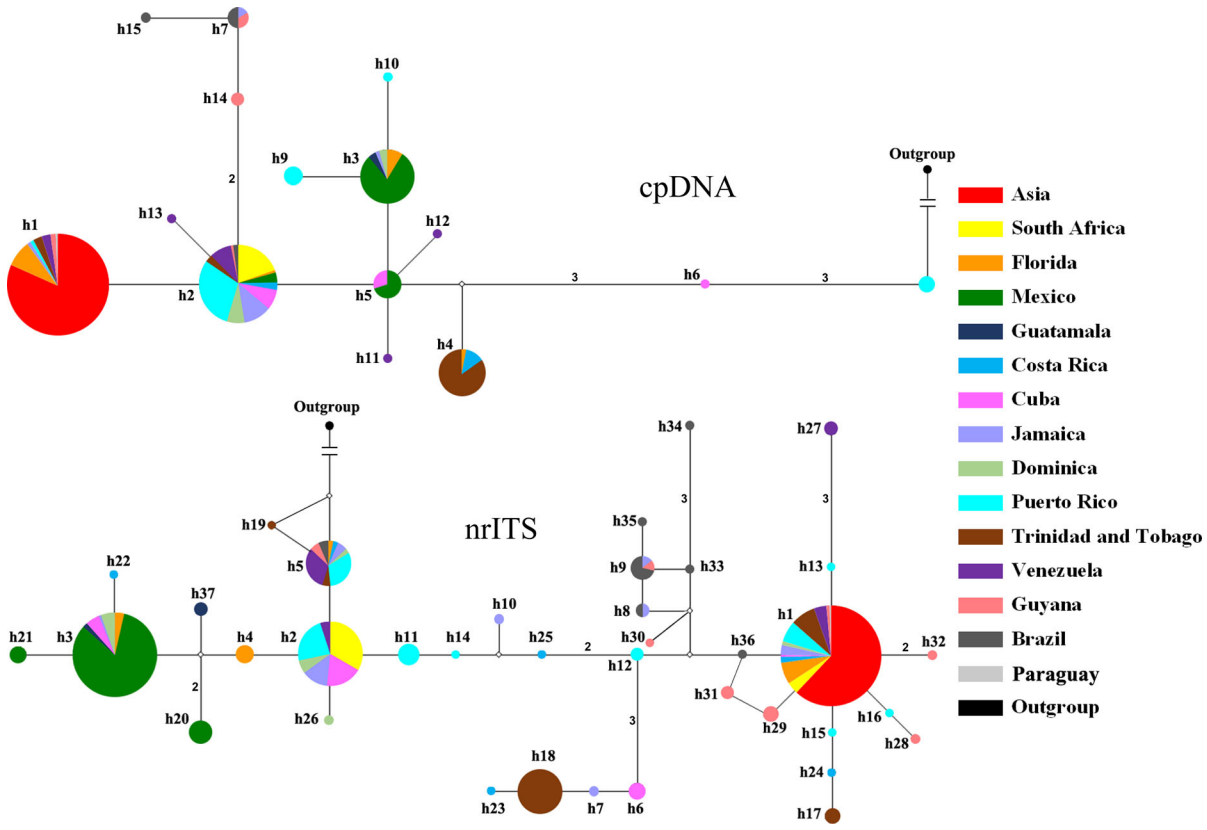

Fig. 2 Network of haplotypes of *Chromolaena odorata* based on cpDNA and ITS. The numbers on branches indicate mutational steps

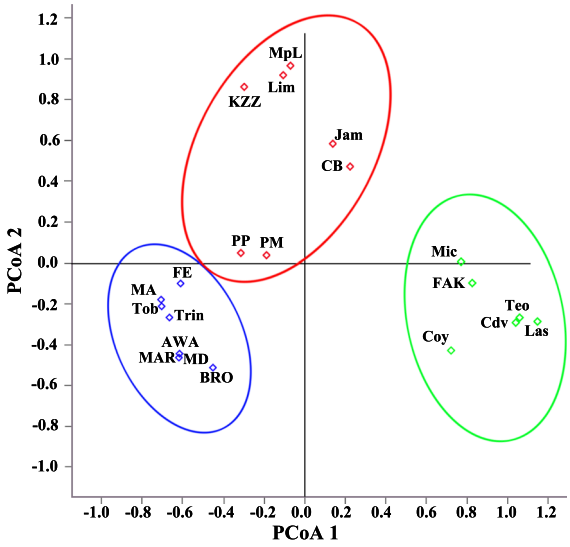

Fig. 3 Principal coordinate analysis (PCoA) of SSR genotype from 21 populations of 558 individuals of *Chromolaena odorata*

heterozygous sites of ITS in SA biotype. Barker et al. (2005) reported similar phenomenon of existing heterozygous sites of ITS in SA biotype, but unknown factors resulting in multiple base sequences in their study made samples scattered throughout the phylogenetic trees, which, however, were contradictory to the morphological (Zachariades et al. 1999), isozymes (Vos 1989) and genetic (Paterson and Zachariades 2013) evidences. Our study produced uniform base sequences that were more in accord with other evidence. Recently, the difference between the two biotypes was also found in the antimicrobial and activity and in vitro toxicity in medicinally use (Omokhua et al. 2016). It was worth noting that the one sample (MpL4) in our research had a homozygous ITS haplotype (h2) that was also reported in Barker et al. (2005). This ITS haplotype (h2) was distinctive to the haplotype of AWA biotype (h1).

The high genetic uniformness of individuals in invasive populations and geographically separation of main invasive genotypes have also been reported in

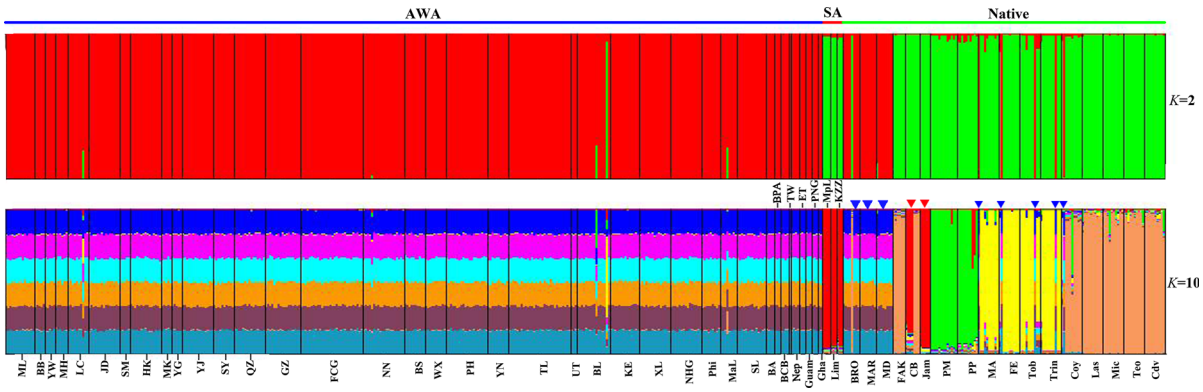

Fig. 4 Bayesian inference analysis of nuclear microsatellite DNA data of *Chromolaena odorata*. Results of both two ($K = 2$) and ten ($K = 10$) clusters were shown; blue arrows marked the invasive genotype found in AWA biotype detected in Florida, Trinidad and Tobago populations; red arrows marked the invasive genotype found in SA biotype detected in Cuba and Jamaica

studies on other invasive plant species (Meimberg et al. 2005), suggesting that successful invaders may not necessarily have high genetic diversity (Poulin et al. 2005; Ren et al. 2005; Geng et al. 2007; Dlugosch and Parker 2008; Perdereau et al. 2013). Low genetic diversity may favour the expression of plastic phenotypes in genetically similar plants colonizing various environments, which could facilitate the adaptation and spread of the invasive species (Meimberg et al. 2005). For example, phytochemicals of mainly alkaloids and steroids from leaves of AWA biotype samples in the Zamboanga Peninsula may alter the reproduction of the used biocontrol insects (McFadyen 2002; Codilla and Metillo 2012). In contrast, plants of AWA biotype in West Africa (Biller et al. 1994) and Thailand (Pisutthanan et al. 2006) contain more flavonoids, while those from India contain all of these phytochemicals (Suriyavathana et al. 2012). Plants AWA biotype also show a vertical grow habitat in open-land habitats and a prostrate creeping (lax grow) habit supported by other vegetation like a jungle, which allowed the species to flourish in an extremely wider range of habitats. AWA biotype appears to be more fire-resistant than SA biotype, which reduced the fire-induced mortality rates (Zachariades et al. 2009). As for *C. odorata* in Africa, superior light interception ability of SA biotype when temporarily released from the competition by disturbance make it the dominant genotype.

The source locations of AWA and SA biotypes

Some researcher believed that the current distribution of AWA biotype spread from the first collection in India (Clarke 1876; Gautier 1992). However historical literature recorded introductions of *C. odorata* from the West Indies to India and Singapore/Malaysia, respectively (Biswas 1934; Rao 1968; McFadyen 1989; Muniappan et al. 2005). In the 1920s, *C. odorata* was introduced as a spice plant for cultivation in Thailand (Yu et al. 2010). In 1989, it was introduced into southern Taiwan as a medicinal plant (Wu et al. 2004; Lai et al. 2006). However, the source of both introductions was unknown. Our results of both the haplotype and MLG analysis revealed that samples from Florida and Trinidad showed an affinity with samples from Asia (Figs. 1, 3, 4), which has also been revealed in Paterson and Zachariades (2013) using ISSRs and Yu et al. (2014) with the same methodology of this study. The genetic similarity of samples from AWA biotype distributions, to some extent, supported the view that *C. odorata* in these regions was probably attributed to a single introduction from Trinidad and Tobago and adjacent West Indies (McFadyen 1989; Goodall and Erasmus 1996; Yu et al. 2014). In addition, it was worth mentioning that the single haplotype (hap-AWA) in AWA group was also found in Jamaica (population JT) (Fig. 1) which was previously assumed as the possible source location of AWA biotype (McFadyen 1993). The previous evidence from Paterson and Zachariades (2013) and our results of DNA sequences analysis both proved that

Venezuela also could be the origin of AWA biotype. The combined evidence from the historical literature and genetic results suggest that Trinidad and Tobago was the most likely origin of AWA biotype, while analysis with more samples from Jamaica and Venezuela were needed for a definite conclusion.

However, it is not clear whether *C. odorata* invaded to Asia by one single introduction from one origin accidentally or multiple invasions intentionally for its benefits, without further investigation and sieving through historical literature. For example, AWA biotype is considered as a traditional medicinal plant widely used for wound-healing and as a fallow species promoting soil restoration (M'Boob 1991; Atagana 2011; Majumder et al. 2014; Pandith et al. 2014; Omokhua et al. 2016), which might have increased the intentional introductions. It needs caution to specify any particular location as source location of AWA biotype, and it may have multiple origins as Paterson and Zachariades (2013) speculated.

Paterson and Zachariades (2013) previously identified Jamaica or Cuba as the possible origin of the SA biotype by using ISSR analysis, which is consistent with results from our analysis using DNA haplotype and SSR analysis. However, our study also revealed that some samples from Puerto Rico and the Dominican Republic, both of which are close to Cuba and Jamaica, were also genetically similar to the SA biotype both in cpDNA and ITS sequences, suggesting that these two regions may also be the possible source locations of SA biotype. Indeed, plants of *C. odorata* from these islands similar to SA biotype in morphology also have been reported (Zachariades 2003; Zachariades et al. 2004). However, the result of SSR in this study failed to testify Puerto Rico to be one of the origins of SA biotype, as samples from the Dominican Republic were not used for the SSR analysis because of the low-quality DNA. Therefore, more samples from both regions are needed for further study.

Implications for biocontrol of *C. odorata*

Populations with low genetic diversity and no novel genotypes generated across invasion are conducive to the success of biocontrol (Ward et al. 2009). Biocontrol is more likely to be successful if the agent is collected from the native plant that is genetically identical to invasive biotypes. Our results suggest that the most appropriate biocontrol agents for AWA biotype should be sourced from Trinidad and Tobago. A moth, *Parachutes pseudoinsulata*, from Trinidad has been the first agent to be successfully established in many countries invaded by AWA (Muniappan and Bamba 2000; Zachariades et al. 2009; Rusdy 2016). However, for the countries where *P. pseudoinsulata* failed to establish, other agents from Trinidad and Tobago were worth exploring.

For SA biotype, the promising source of the agent may be from Cuba and Jamaica. Additionally, Puerto Rico, Dominica and Venezuela may also hold the promise as the results of DNA sequences analysis indicated. Agents from Guam (Kluge 1994), Venezuela and other countries where plants are different morphologically with SA biotype were introduced to South Africa, while they all failed because of complex reasons (including plant–insect incompatibility) (Zachariades et al. 2011). A leaf-eating insect, *Pareuchaetes insulata*, collected from populations in Florida, has been released in 2001 to patchy areas in KwaZulu-Natal where the SA biotype are present (Zachariades et al. 2011). In 2003, populations of *P. insulata,* collected from Jamaica and Cuba, were released to other patches (Zachariades et al. 2011). In December 2004, a small outbreak of *P. insulata* larvae was unexpectedly found near a site where Florida population released and other outbreaks followed intermittently. However, the origin of the successful agent was not clear as one site where Jamaica population was released also included in these outbreaks (Zachariades et al. 2011, 2016). Nevertheless, these studies showed the promise of biocontrol of SA biotype of *C. odorata* in South Africa. *P. insulata* can be expected to establish in other distributions where SA biotype is a threat. However, there is another potential problem for *P. insulata* as an effective biocontrol agent across wide regions. *P. insulata* is a leaf-eating insect, the place where plants drop their leaves in the cool and dry season would prevent the persistence of this agent (Zachariades et al. 2016). This problem also appears to affect another potential agent from Jamaica, the leaf-mining fly *Calycomyza eupatorivora* Spencer (Diptera: Agromyzidae) which has established along the coastal section invaded by *C. odorata* (Zachariades et al. 2011). Further efforts are needed to identify other agents that are not confined by seasonal availability of food from the host *C. odorata*. Our results suggest that

agents from Cuba and Jamaica where SA biotype are present, and from other native regions with dry and cold microenvironment similar to habitats invaded by *C. odorata* are needed for biocontrol of SA biotype in South Africa.

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