MOLECULAR ECOLOGY

Molecular Ecology (2010) **19**, 1774–1786 doi: 10.1111/j.1365-294X.2010.04609.x

Genetic uniformity characterizes the invasive spread of water hyacinth (*Eichhornia crassipes*), a clonal aquatic plant

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Abstract

Aquatic plant invasions are often associated with long-distance dispersal of vegetative propagules and prolific clonal reproduction. These reproductive features combined with genetic bottlenecks have the potential to severely limit genetic diversity in invasive populations. To investigate this question we conducted a global scale population genetic survey using amplified fragment length polymorphism markers of the world's most successful aquatic plant invader – *Eichhornia crassipes* (water hyacinth). We sampled 1140 ramets from 54 populations from the native (South America) and introduced range (Asia, Africa, Europe, North America, Central America and the Caribbean). Although we detected 49 clones, introduced populations exhibited very low genetic diversity and little differentiation compared with those from the native range, and ~80% of introduced populations were composed of a single clone. A widespread clone ('W') detected in two Peruvian populations accounted for 70.9% of the individuals sampled and dominated in 74.5% of the introduced populations. However, samples from Bangladesh and Indonesia were composed of different genotypes, implicating multiple introductions to the introduced range. Nine of 47 introduced populations contained clonal diversity suggesting that sexual recruitment occurs in some invasive sites where environmental conditions favour seedling establishment. The global patterns of genetic diversity in *E. crassipes* likely result from severe genetic bottlenecks during colonization and prolific clonal propagation. The prevalence of the 'W' genotype throughout the invasive range may be explained by stochastic sampling, or possibly because of pre-adaptation of the 'W' genotype to tolerate low temperatures.

Keywords: aquatic invasions, AFLP markers, clonal propagation, *Eichhornia crassipes*, genetic bottlenecks

Received 9 December 2009; revision received 22 February 2010; accepted 23 February 2010

Introduction

Biological invasions are non-equilibrium demographic processes involving migration and recurrent colonization episodes associated with the occupation of new territory. Historical contingency and stochastic processes are expected to play a prominent role in shaping the patterns of genetic diversity in the introduced range of many invasive plants owing to their diverse reproductive modes (Barrett & Husband 1990; Novak & Mack 2005; Keller & Taylor 2008; Barrett *et al.* 2008). The analysis of patterns of genetic diversity among native and introduced populations can give insight into historical processes associated with colonization history, including founder events, genetic bottlenecks and the occurrence of multiple introductions (Taylor & Keller 2007; Dlugosch & Parker 2008). Such analyses can also aid in determining the relative importance of stochastic vs. deterministic forces in shaping patterns of genetic diversity in invasive species. In addition, identification of the geographic origin(s) of invasions enables direct

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ancestor-descendent comparisons of phenotypic traits between native and introduced populations (Keller & Taylor 2008; Prentis *et al.* 2008; Colautti *et al.* 2009). Surveys of genetic diversity and reconstruction of the introduction history of invasive populations are useful first steps towards understanding the ecological and evolutionary processes underlying invasive success.

Invasive plants exhibit diverse modes of reproduction including variation in the relative importance of sexual vs. asexual reproduction, and the degree of outcrossing vs. selfing, both of which have a significant impact on the demography and genetic structure of populations (Sakai *et al.* 2001; Barrett *et al.* 2008; Barrett in press). Uniparental reproduction (e.g. selfing or asexual reproduction) during colonization can provide reproductive assurance when mating partners are rare (reviewed in Eckert *et al.* 2006). However, uniparental reproduction restricts recombination and opportunities for genetic admixture, which can magnify founder effects and increase the likelihood of stochastic processes influencing patterns of genetic diversity (Golding & Strobeck 1980; Husband & Barrett 1991; Kliber & Eckert 2005). Stochastic forces associated with long-distance dispersal can also result in a restricted sampling of mating types among founder groups, and this has the potential to disrupt reproductive systems of invasive plants. Shifts to asexual propagation have occurred in several clonal invaders because of the absence of sexual partners in the introduced range (e.g. *Elodea canadensis,* Sculthorpe 1967; *Fallopia japonica,* Hollingsworth & Bailey 2000; *Oxalis pes-caprae,* Ornduff 1987). Hence, colonization history can play a critical role in determining modes of reproduction and patterns of genetic diversity in the introduced range of invasive plants.

The water hyacinth, *Eichhornia crassipes* (Mart.) Solms (Pontederiaceae), is a clonal aquatic plant that has become the world's most noxious invader of wetland environments (Holm *et al.* 1977; Gopal & Sharma 1981; Barrett 1989) during the past 150 years. It is native to South America, primarily Brazil and Argentina, but has spread to more than 50 countries, including tropical, subtropical and temperate zones on five continents. Today, *E. crassipes* is on the IUCN's list of the 100 most dangerous invasive species (Tellez *et al.* 2008). Several attributes of *E. crassipes* have contributed to its success as a wordwide invader of aquatic habitats. These include a prolific capacity for multiplication through clonal reproduction, the high mobility of its free-floating life form and very high rates of growth under appropriate environmental conditions (Penfound & Earle 1948; Sculthorpe 1967). Significantly, these features are not represented among the remaining seven species of *Eichhornia*, which are also aquatic, but have not become serious weeds (Barrett 1992).

The introduction history of *E. crassipes* to various regions is difficult to document accurately. Scattered evidence indicates that plants collected from the lower Orinoco River in Venezuela were distributed as gifts at a cotton exposition in New Orleans, USA in 1884 but soon 'escaped' to various localities in North America and may also have been taken overseas (reviewed in Sculthorpe 1967; Gopal & Sharma 1981; Barrett 1989). Its first appearance in Asia was reported from the Bogor Botanical Garden in Indonesia in 1894. Specimens discarded into a river soon became a nuisance and many rivers in the region subsequently became infested with the large floating mats that characterize the species. In Africa, *E. crassipes* was first introduced to Egypt and Sudan in the later part of the 19th century and is now widespread throughout the continent (Mendonca 1958; Sculthorpe 1967). In Europe, *E. crassipes* appears to have been first introduced to Portugal in 1939 as an ornamental; since then it has spread over the central-west of the country and is currently distributed in the middle and lower Sado and Tagus basins (Tellez *et al.* 2008). However, the migration history of water hyacinth and the origins of source populations in most locations that it occurs in today remain largely unknown.

Eichhornia crassipes possesses the genetic polymorphism tristyly in which three floral morphs occur (long-, mid- and short-styled, hereafter L-, M- and S-morphs) that differ in style length and anther height (Barrett 1977). The geographical distribution of floral morphs indicate that founder events have played a prominent role in the species worldwide spread (Barrett 1989). Tristylous populations are confined to lowland South America; whereas in the introduced range the M-morph predominates, with the L-morph occurring infrequently. Hence, the S-morph is absent altogether from the Old World and the New World adventive range and until relatively recently was not known to exist (Barrett 1977). Mulcahy (1975) conjectured that due to extensive clonal propagation and a limited capacity for sexual reproduction, a small number of genotypes, perhaps only one, might be represented in parts of the introduced range of *E. crassipes*. Two recent surveys using random amplified polymorphic DNA (RAPDs) and inter-simple sequence repeats (ISSRs) markers have provided support for this hypothesis. The genetic diversity of *E. crassipes* in southern China was extremely low (Ren *et al.* 2005; Li *et al.* 2006). However, sampling of genetic diversity in other parts of the adventive range has not been conducted so it is unclear how general these results are. In addition, apart from a large survey of floral morphs (Barrett & Forno 1982), nothing is known about the patterns of diversity in the native range of *E. crassipes.*

Here we use amplified fragment length polymorphisms (hereafter AFLPs) to investigate genetic diversity and population genetic structure of *E. crassipes* in the species' native and introduced range. AFLP markers are especially suitable for studies of intraspecific variation, where it may be necessary to amplify many loci to identify the few that are polymorphic (Hilde 2004; Meudt & Clarke 2007). Our study had three main objectives: (i) to corroborate whether the worldwide spread of *E. crassipes* has been associated with founder events and a loss of diversity compared with the native range; (ii) to investigate if colonization of the introduced range involved more than one introduction; (iii) to evaluate whether populations in the introduced range are reproducing exclusively by clonal propagation. Following the presentation of our results we discuss the role of historical contingency in the invasion process, compare our results to others conducted on invasive and non-invasive clonal species, and consider the role of ecological and reproductive factors in affecting geographical patterns of genetic diversity.

Materials and methods

Sampling

During 2003–2009 we collected 1140 samples of *E. crassipes* leaf tissue from 54 native and introduced populations, with a particular focus on China where the species occurs over an extensive geographical area (Table 1). In each population, we sampled a single leaf from individual ramets separated by at least one meter. The mean number of ramets sampled in each population was 21.1, range 1–41. Leaves were dried with silica gel in zip-lock bags and preserved at room temperature. Because most populations of *E. crassipes* are highly clonal, it was impossible to sample at the genet level, especially in populations that contained only a single floral morph, the commonest condition that we encountered.

DNA extraction and AFLP genotyping

We extracted total genomic DNA from ~ 10 to 30 mg of dried leaf tissue using a plant genomic DNA Kit (Tiangen, Beijing, China). We performed AFLP genotyping according to the original protocol of Vos *et al.* (1995) with several modifications. Restriction and ligation were performed during a single step in an 20 μL reaction containing 200–400 ng genomic DNA, 8 U *Eco*RI [New England Biolabs (NEB)], 2 U *Mse*I (NEB), 80 U T4 DNA ligase (NEB), 1 X NEBuffer2 (50 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris–HCl, 1 mM DDT), 2 μg BSA, 0.2 mM ATP, 0.2 μM *Eco*RI-adaptor, 0.16 μM *Mse*I-adaptor and H_2O. We incubated the restriction and ligation reaction in a PTC-200 thermal cycler (MJ Research Inc) at 37 °C for 3 h, and denatured at 70 °C for 10 min. We performed a pre-selective polymerase chain reaction (PCR) in a 20 μL reaction containing 2 μL restriction-ligation product, 0.5 U r*Taq* polymerase (TaKaRa), 1 X PCR buffer, 2.0 mM $MgCl_2$, 0.25 mM each dNTP (TaKaRa), 0.25 μM of each pre-selective amplification primer (E-A and M-C) and H_2O. The pre-selective PCR was performed on a PTC-200 at 94 °C for 2 min, followed by 28 cycles of 94 °C for 45 s, 56 °C for 45 s, 72 °C for 1 min, and finishing with an extension at 72 °C for 10 min. We diluted 5 μL of the pre-selective PCR product (20 times) into 95 H_2O and selective amplification was performed in a 20 μL reaction containing 3 μL of the diluted pre-selective PCR product, 0.5 U r*Taq* polymerase, 1 X PCR buffer, 2.0 mM $MgCl_2$, 0.25 mM each dNTP, 0.25 μM of *Eco*RI selective primer (E-ANN tagged with 6-FAM), 0.30 μM of *Mse*I selective primer (M-CNN) and H_2O. The selective PCR was performed at 94 °C for 2 min, followed by 13 cycles of 94 °C for 30 s, 65 °C for 30 s (decreasing by 0.7 °C each cycle), 72 °C for 1 min, 23 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min and finishing with 72 °C for 5 min. 0.5 μL of each selective PCR product and 0.3 μL ROX-500 (Applied Biosystems) were denaturalized in 9.2 μL formamide at 94 °C for 4 min, and immediately at −20 °C for 5 min. We loaded the denatured mixtures on an ABI 3100 automated Genetic Analyser (Applied Biosystems) to assess fragment sizes.

We performed primer combination screening on a subset of 16 samples (each from a different population). After screening 40 combinations, we chose four primer pairs (E-AAC/M-CAC, E-AAC/M-CTG, E-ACT/M-CAT, E-ACT/M-CTT) following Meudt & Clarke (2007), which all yielded well-separated peaks, clear polymorphism and a moderate number of bands distributed throughout the available size range. This reduced errors in subsequent scoring, as well as the probability of homoplasy (Vekemans *et al.* 2002).

We scored the AFLP profiles using Genemapper software version 3.7 (Applied Biosystems). The size range of markers, locus selection threshold and phenotype-calling threshold were considered critical scoring parameters (see Holland *et al.* 2008; Whitlock *et al.* 2008). In our analysis, we included peaks within the size range of 70–500 bp. To avoid ambiguities, only peaks with intensity (or relative fluorescence units, rfu) exceeding 200 were included as markers. Our phenotype-calling threshold for markers was chosen to be around 10% of the highest peak's intensity, above which we scored peaks as 'present' (1) or below as 'absent' (0), following Bonin *et al.* (2004). Genemapper automatically generated the binary matrix according to

Table 1 Location, sample sizes and population genetic parameters for the 54 populations of *Eichhornia crassipes* sampled in this study. *N* = number of ramets sampled in each population; *G* = number of distinct clones (genotypes); *R* = proportion of distinguishable genotypes; NPL = number of polymorphic loci at 5% level

Region	Population	Pop ID	Location		*N*	*G*	*R*	*He*	NPL	Genotype ID
Native range										
South America	Rosario, Argentina	Ar. R	32° 57′ S	60° 40′ W	3	3	1.000	1.000	44	Ar.R-S(l), -M(l), -L(l)
	Central Garden, Campinas, Brazil	Br. Cl	22° 52′ S	47° 03′ W	23	1	0.000	0.000	–	Br.Cl(23)
	Inst. Agriculture, Campinas, Brazil	Br. C2	22° 54′ S	47° 04′ W	11	1	0.000	0.000	–	Br.C2(ll)
	Indian village, Manaus, Brazil	Br. Ml	3° 07′ S	59° 53′ W	10	4	0.333	0.644	88	Br.M2(6),3(2), 6(l),7(l)
	Rain forest, Manaus, Brazil	Br. M2	3° 06′ S	60° 03′ W	31	5	0.133	0.351	66	Br.Ml(25),2(l), 3(2),4(2),5(l)
	Iquitos, Peru	Pe. I	3° 47′ S	73° 18′ W	14	4	0.231	0.495	63	W(10),P.I1(2), 2(1),3(1)
	Puerto Maldonado, Peru	Pe. P	12° 37′ S	69° 12′ W	12	1	0.000	0.000	–	W(12)
Sum of native range		7			104	16			123	
Invasive range										
Asia	Mymensingh, Bangladesh	B. M	24° 45′ N	90° 24′ E	25	1	0.000	0.000	–	B.M(25)
	Chengdu, China	C. CD	30° 37′ N	104° 05′ E	27	1	0.000	0.000	–	W(27)
	Chongqing, China	C. CQ	29° 37′ N	106° 31′ E	36	8	0.200	0.398	23	W(24),C.CQ1(2), 2(1),3(1), 4(1),5(1),6(1),7(1)
	Fuzhou, China	C. FZ	26° 01′ N	119° 23′ E	25	1	0.000	0.000	–	W(25)
	Guangzhou, China	C. GZ	23° 05′ N	113° 21′ E	29	1	0.000	0.000	–	W(29)
	Haikou-1, China	C. HK1	20° 00′ N	110° 16′ E	31	1	0.000	0.000	–	W(31)
	Haikou-2, China	C. HK2	19° 59′ N	110° 21′ E	28	1	0.000	0.000	–	W(28)
	Hanyang, China	C. HY	30° 39′ N	114° 17′ E	24	1	0.000	0.000	–	W(24)
	Hangzhou, China	C. HZ	30° 17′ N	120° 05′ E	24	1	0.000	0.000	–	W(24)
	Kunming, China	C. KM	25° 00′ N	102° 38′ E	31	2	0.033	0.323	12	W(25), C.KM(6)
	Nanchang, China	C. NC	28° 13′ N	115° 55′ E	24	1	0.000	0.000	–	W(24)
	Nanning-1, China	C. NN1	22° 51′ N	108° 14′ E	29	4	0.107	0.534	27	C.NN1(18), 2(9), 3(1), 4(1)
	Nanning-2, China	C. NN2	22° 50′ N	108° 16′ E	28	4	0.111	0.578	26	W(l), C.NN1(15), 5(11),6(1)
	Nanning-3, China	C. NN3	22° 48′ N	108° 15′ E	29	1	0.000	0.000	–	C.NN7(29)
	Panjin, China	C. PJ	40° 57′ N	122° 03′ E	23	1	0.000	0.000	–	W(23)
	Shanghai, China	C. SH	31° 10′ N	121° 18′ E	27	1	0.000	0.000	–	W(27)
	Taizhong, Taiwan, China	C. TZ	24° 06′ N	120° 40′ E	22	1	0.000	0.000	–	W(22)
	Xiamen, China	C. XM	24° 28′ N	118° 05′ E	31	5	0.133	0.490	27	W(22), C.XM1(3), 2(2), 3(2), 4(2)
	Xishuangbanna, China	C. XS	22° 00′ N	100° 49′ E	29	1	0.000	0.000	–	W(29)
	Yunlin, Taiwan, China	C. YL	23° 41′ N	120° 31′ E	30	1	0.000	0.000	–	W(30)
	Cibinong, Indonesia	I. C	06° 29′ S	106° 50′ E	34	2	0.030	0.499	36	W(14),I.C(20)
	Semarang, Indonesia	I. S	07° 16′ S	110° 27′ E	41	6	0.125	0.689	63	W(21),I.S1(8),I.S2(5), I.S3(4),I.S4(2),I.S5(1)
	Nagoya, Japan	J. N	35° 11′ N	136° 52′ E	28	1	0.000	0.000	–	W(28)
	Seto, Japan	J. S	35° 13′ N	137° 05′ E	1	1	0.000	0.000	–	W(l)
	Colombo, Sri Lanka	SL. C	6° 54′ N	79° 51′ E	28	3	0.074	0.442	11	W(7),SL.C(20), Br.C1(1)
	Kandy, Sri Lanka	SL. K	7° 18′ N	80° 39′ E	21	1	0.000	0.000	–	W(21)
	Cau Giay, Ha Noi, Vietnam	V. HN1	21° 02′ N	105° 50′ E	5	1	0.000	0.000	–	W(5)
	Gia Lam, Ha Noi, Vietnam	V. HN2	21° 01′ N	105° 55′ E	35	1	0.000	0.000	–	W(35)
	Ha Tay, Vietnam	V. HT	21° 53′ N	105° 38′ E	21	1	0.000	0.000	–	W(21)
	Nam Dinh, Vietnam	V. N	21° 25′ N	106° 11′ E	20	1	0.000	0.000	–	W(20)

Table 1 (*Continued*)

Region	Population	Pop ID	Location		*N*	*G*	*R*	*He*	NPL	Genotype ID
Africa	Nairobi Dam, Kenya	K. Nr	1° 19′ S	36° 48′ E	30	1	0.000	0.000	–	K.Nr(30)
	Lake Naivasha, Kenya	K. Nv	0° 24′ S	36° 17′ E	31	1	0.000	0.000	–	W(31)
	Kisumu, Kenya	K. K	0° 06′ S	34° 45′ E	25	2	0.042	0.080	2	W(24), K.K(1)
	Uyoma, Kenya	K. U	0° 05′ S	34° 44′ E	19	1	0.000	0.000	–	W(19)
Europe	Ribatejo, Portugal	P. R	38° 45′ N	9° 08′ W	29	1	0.000	0.000	–	W(29)
	Badajoz, Spain	S. B	38° 53′ N	6° 58′ W	33	1	0.000	0.000	–	W(33)
	Estremadura, Spain	S. E	39° 29′ N	6° 03′ W	32	1	0.000	0.000	–	W(32)
North America	Tamiami Trail, Florida, USA	A. F	25° 54′ N	81° 02′ W	6	1	0.000	0.000	–	A.F(6)
	Honolulu, Hawaii, USA	A. H	21° 18′ N	157° 49′ W	1	1	0.000	0.000	–	A.H(1)
Central America	Nassau, Bahamas	Ba.N	25° 04′ N	77° 20′ W	5	1	0.000	0.000	–	W(5)
	Sierpe, Costa Rica	CR. S	8° 52′ N	83° 28′ W	3	1	0.000	0.000	–	W(3)
	Guantanamo, Cuba	Cu. G	23° 04′ N	82° 22′ W	6	1	0.000	0.000	–	Cu(6)
	Black River , Jamaica	Ja. B	18° 02′ N	77° 50′ W	6	1	0.000	0.000	–	W(6)
	Ferris Cross, Jamaica	Ja. F	18° 14′ N	78° 04′ W	6	1	0.000	0.000	–	Ja(6)
	Little London, Jamaica	Ja. L	18° 15′ N	78° 12′ W	6	1	0.000	0.000	–	W(6)
	Sandy Ground, Jamaica	Ja. S	18° 05′ N	77° 58′ W	6	1	0.000	0.000	–	Ja(6)
	Panama Canal , Panama	Pa. C	9° 01′ N	79° 36′ W	6	1	0.000	0.000	–	W(6)
Sum of invasive range	47			1036	35			82		
Sum of all	54			1140	49			131		

our parameter settings. Finally, we modified this matrix by checking it against each AFLP profile by eye.

To detect unreliable markers and to estimate the error rate of genotyping (Bonin *et al.* 2004) we replicated 46 randomly chosen DNA samples for a second round of AFLP genotyping. We then compared the results with the original data matrix. Additionally, the same set of samples was chosen as a positive control and were run from the beginning of the restriction-ligation procedure to detect 'technical differences' caused by restriction and/or PCR artifacts and to calibrate the fluorescent signal.

Data analysis

Genotyping error rates

We estimated AFLP total genotyping error rates, following Bonin *et al.* (2004), by comparing the binary matrix of 46 samples and their replicates. The total error rate was calculated as the proportion of differences of all phenotypic comparisons. We estimated the error rate per marker as the total number of differences summed over all samples. We calculated the error rates per AFLP phenotype (multilocus genotype) as the total difference between the phenotype and its replicate summed over all markers. Our analysis obtained 233 AFLP markers from four primer combinations, after excluding seven markers that had high error rates (>10 errors per marker). We excluded unreliable markers with high error rates from subsequent analysis. 58.7% of the replicated samples contained at least one error. The final error rate based on the 46 replicates was reduced from 2.75% to 1.33%.

Clonal membership assignment

Samples from the same clone may give slightly different AFLP phenotypes due to somatic mutations and/or scoring errors and this can lead to an overestimate of the number of clones. We therefore adopted methods for determining clonal membership of each sample, following Douhovnikoff & Dodd (2003) and Meirmans & Tienderen (2004). The algorithm we used calculates genetic distances between each pair of samples, the distribution of which is employed to define the assignment threshold. The existence of somatic mutations or scoring errors may result in a peak at low genetic distances, resulting in bimodal or multimodal distributions. Additional peaks may also represent genetic distances between closely related genets, which can arise from selfing or biparental inbreeding (Meirmans & Tienderen 2004). It has been suggested that the threshold be bounded between the maximum distance among samples from the same genet, and the minimum distance among samples from different related genets, and is identified as the valley between the first two peaks (Douhovnikoff & Dodd 2003). We therefore assigned samples to the same clone if their distances were no larger than this threshold, and considered samples different clones if their distances exceeded that value (see Douhovnikoff & Dodd 2003; Meirmans & Tienderen 2004; Arnaud-Haond *et al.* 2005, 2007;

Rozenfeld *et al.* 2007). We assigned membership to clonal lineages using GenoDive (Meirmans & Tienderen 2004) and set the distance option as 'infinite allele model' equal to the simple mismatch distance (Smouse & Peakall 1999).

The frequency distribution of pairwise genetic distances followed a multimodal distribution (Fig. 1), with six peaks and a very high peak in the low distance category (0–6). Although valley 6 may be considered a good candidate for the clonal assignment threshold, we found that samples from different floral morphs (L- and M-morphs) were assigned to the same clone in a population from Chongqing, China. Thus, we considered the threshold of six conservative and chose instead valley five as our threshold for clonal membership. Using this threshold, all cases involving different floral morphs within a population were identified as different clones (see Table 1). The robustness of threshold five is supported by the fact that thresholds four and five gave the same assignment result (Fig. 1).

It is also possible for different clones to exhibit identical AFLP phenotypes if the markers have low polymorphism and hence there is insufficient resolution. Therefore, it was important to determine if our markers were able to discriminate genotypes of different zygotic origins. This was undertaken by ranking polymorphism for each primer combination over all samples. We then calculated the number of clones that could be discriminated (*G*), using the data matrix generated by the most polymorphic primer pair. We then added data from the second most polymorphic primer pair to the matrix and re-calculated *G* and this process was repeated until *G* levelled off. The plateau, which appeared after adding the second most polymorphic primer pair (Fig. 2), indicates that adding markers or primer pairs does not significantly increase the number of clones identified. Therefore, the markers we used were capable of separating samples from different zygotic origins, even among close relatives.

Analysis of clonal diversity

We use the terms clonal lineage, clone and genotype as synonyms although we recognize that they can refer to different genetic entities (see Arnaud-Haond *et al.* 2007). We calculated the number of distinguishable clones per population (*G*) using GenoDive (Meirmans & Tienderen 2004). We also estimated for each population the proportion of distinguishable genotypes *R*, corrected for monoclonal populations as:

$$(\mathrm{G}-1)/(N-1) \tag{1}$$

where *N* represents the total number of individuals sampled (Dorken & Eckert 2001). This correction ensures that the minimum possible value in monoclonal populations is zero, and the maximum value is one, where each sample is assigned to a distinct clone. We calculated Simpson's index of diversity (*He*) corrected for finite sample size for each population as:

$$\frac{N}{N-1}\left(1-\sum_{i=1}^{G} p_i^2\right), \tag{2}$$

where p_i is the proportion of individuals assigned to the clone (*i*), also known as Nei's (1987) genotypic diversity or expected heterozygosity. We estimated the number of polymorphic markers at the 0.05 level of polymorphism using AFLP-SURV 1.0 (Vekemans *et al.* 2002). The 0.05 level of polymorphism indicates that the frequency of band '1' of a given maker lies within the range of 0.05–0.95. In some cases, we included markers that were polymorphic at the 0.01 level (band '1' frequency between 0.01 and 0.99). This was because some markers were only 'present' in the native range in a small proportion of samples, whereas they were 'absent' in most of the samples from the introduced range. Although these markers were of low frequency they were important for differentiating native and introduced genotypes.

Analysis of genotype clustering

To present graphically the relationships among different clones we used two clustering methods, a tree based analysis and multivariate methods. We calculated the pairwise genetic distance among distinct clones as the simple mismatch distance (Smouse & Peakall 1999), as used in clonal membership assignment. We then used a neighbour-joining (NJ) tree based on this distance matrix, which was constructed using PAUP*4.0b10 (Swofford 2003). We estimated support for branches from 1000 bootstrap replicates. We conducted principal coordinate analysis (PCoA) using Genalex6 (Peakall & Smouse 2006).

Results

Detecting clonal diversity

The discriminative power of the AFLP markers included in our analysis was sufficient to reveal moderate clonal diversity. Of the 233 markers included, 147 (63.1%) were polymorphic at the 0.01 level and 68 (29.2%) at the 0.05 level. We ranked the primer combinations according to their polymorphism at the 0.05 level (Fig. 2a). The number of polymorphic markers ranged from 15 (26.3%) to 22 (41.5%) The most polymorphic primer pair distinguished 42 clones, and this rose to 47 when the second most polymorphic pair was

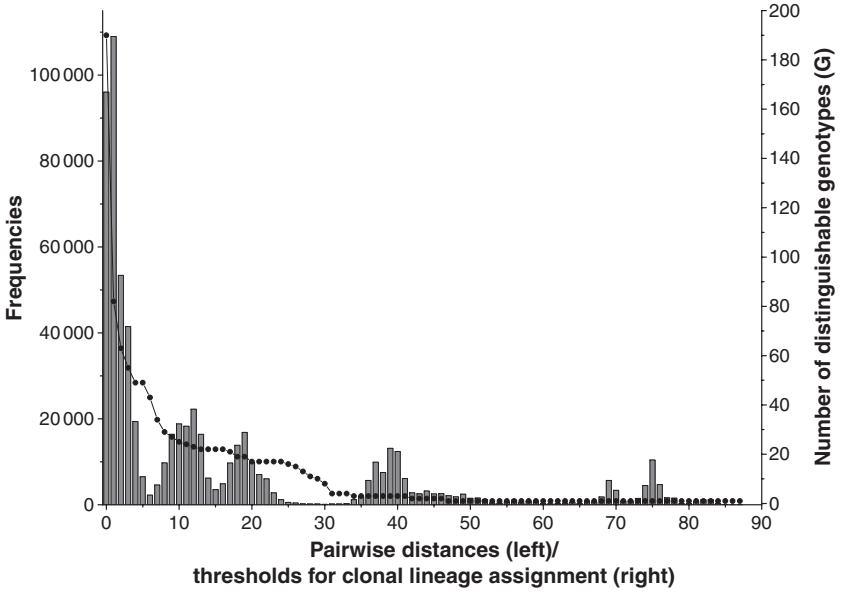

Fig. 1 Frequency distribution of pairwise genetic distances based on AFLP markers among all *Eichhornia crassipes* samples (left axis) and number of distinguishable clones under a given threshold (right axis). Pairwise distance was calculated as simple-mismatch distance between two AFLP phenotypes. The distribution of pairwise distances follows a multimodal distribution with a high peak towards the low distances. Black circles indicate the number of distinguishable clones under a given threshold. The threshold defines the maximum genetic distance between two samples assigned to the same clone. The number of distinguishable clones levels off at the threshold from four to five. We chose five as the threshold and the 1140 samples were assigned to 49 distinct clonal lineages.

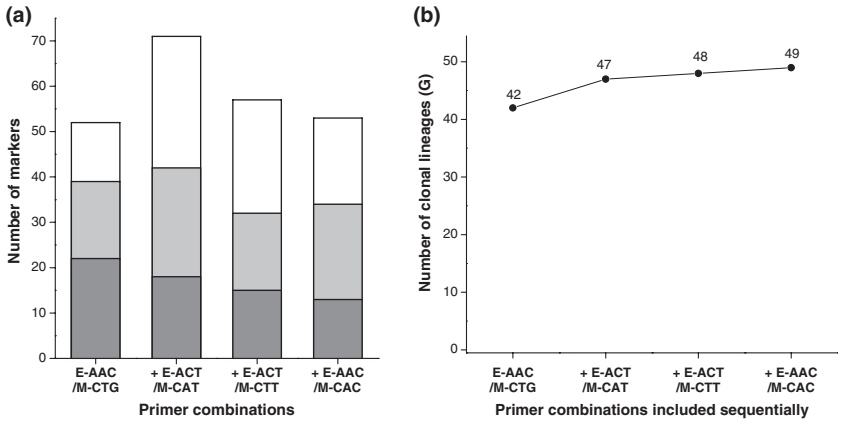

Fig. 2 (a) Polymorphism of each AFLP primer combination. Dark grey represents the number of polymorphic markers at 0.05 level for all *Eichhornia crassipes* samples from a given primer pair, light grey represents number of polymorphic markers at 0.01 level, and white indicates the number of monomorphic markers. We ranked the primer combinations according to their polymorphism at the 0.05 level. (b) The ability of AFLP markers to distinguish different clones of *Eichhornia crassipes*. E-AAC/M-CTG is the most polymorphic primer combination at the 0.05 level, and this pair is capable of discriminating 42 clones. By adding the second most polymorphic pair the number of distinguishable clones increased to 47. Including the next two pairs increases this number by two. Our analysis indicates that 233 AFLP markers are sufficient to distinguish samples of different zygotic origins.

included (Fig. 2b). The gain in distinguishing clones levelled off with additional primer pairs increasing *G* slightly from 47 to 49.

Using an assignment threshold of five, we detected a total of 49 clones from the 1140 samples of *E. crassipes*. There was striking variation in the abundance of individual clones in our sample. Twelve clones accounted for 91.9% of the total sample whereas 20 clones were represented by a single sample. Significantly, we detected a single widespread clone (hereafter 'W'), which accounted for 70.9% of all samples. This clone dominated in 74.5% of all populations and occurred throughout the invasive range and also in two populations from Peru (Table 1, Fig. 3). Clone 'W' was also found in several populations where it was not the most abundant clone (e.g. Nanning-2, China; Cibinong, Indonesia; Colombo, Sri Lanka). The large variation in the abundance of clones, and particularly the dominance of clone 'W', probably explains why we observed multiple peaks in the histogram of pairwise distances (Fig. 1).

Geographical patterns of clonal diversity

The majority of populations in the introduced range of *E. crassipes* were genetically uniform. Of the introduced

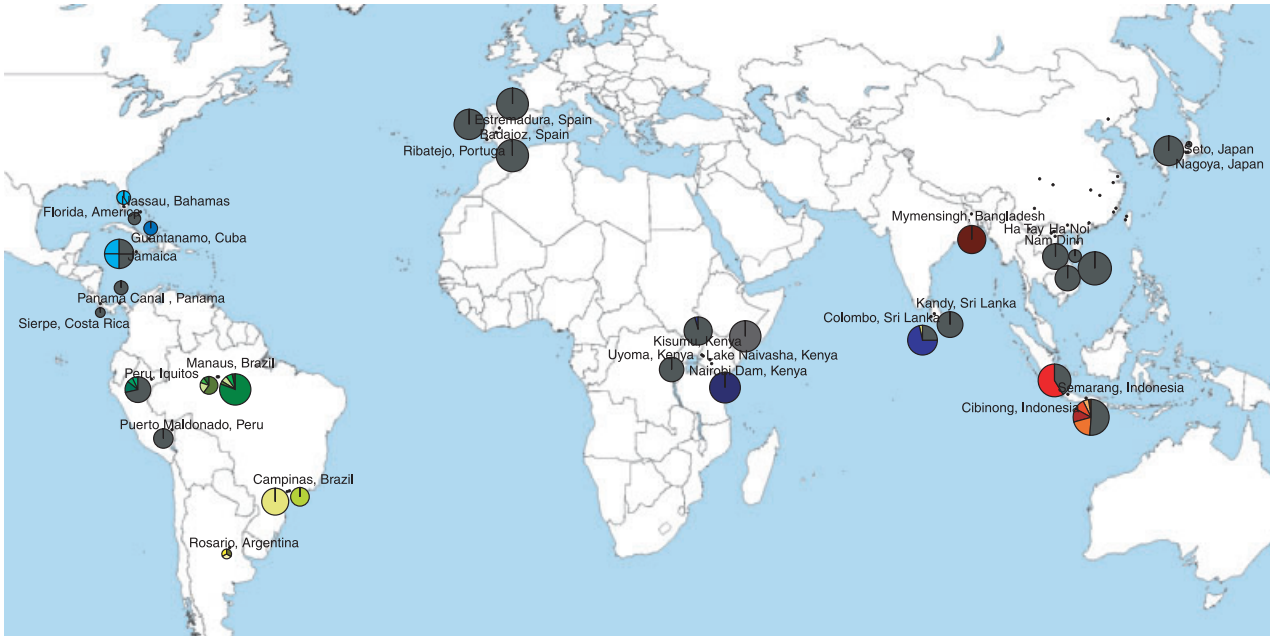

Fig. 3 The global sample of *Eichhornia crassipes* clones in this study including both the native and introduced range. The 1140 samples were assigned to 49 distinct genotypes based on AFLP markers. Different colours in the pie diagrams represent different genotypes. The size of each pie represents the sample size (number of ramets) and colours indicate the proportion of each clone in the sample. Note where multiple populations were sampled for a particular location (e.g. Jamaica) these are pooled. This figure shows the prevalence of 'W' (grey) in introduced populations and its occurrence in two Peruvian populations (Iquitos and Puerto Maldonado). Because of the higher intensity of sampling in China the data are provided in Fig. 4.

populations (n = 47) that had more than one sample (n = 45), 80.0% contained a single clone (Table 1, Fig. 3) and only nine (20.0%), occurring in China, Indonesia and Sri Lanka, had more than one clone. This pattern was also evident at a regional scale in China where more intensive sampling of populations was conducted. Clone 'W' dominated over much of the Chinese range, although five populations (Chongqing, Kunming, Nanning-1 and Nanning-2, Xiamen) contained differing amounts of clonal diversity (Fig. 4). Despite the relatively small sample of native populations, four of the seven were polymorphic. For polymorphic populations, the proportion of distinguishable genotypes (R) ranged from 0.03 to 0.20 in the introduced range, and 0.13 to 0.33 in the native range. Similarly, the number of polymorphic loci in the sample of introduced populations was low (<30) compared to native populations (63–88), except for the two populations from Indonesia, which exhibited higher levels of polymorphism (36, 63)

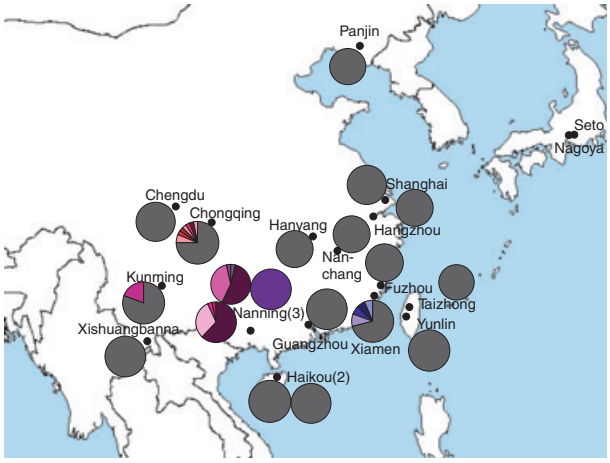

Fig. 4 The distribution of *Eichhornia crassipes* clones throughout China. Different colours represent different genotypes. The size of each pie represents the sample size (number of ramets) and the colours indicate the proportion of each clone in the sample. Five populations in China (Chongqing, Kunming, Nanning-1, 2 and Xiamen) contained more than one genotype suggesting that sexual recruitment occurs in these populations.

Genetic relationships among clones

Using a neighbour-joining tree the 49 clones of *E. crassipes* clustered into three main groups (1,2,3) corresponding to bootstrap values of 97, 76 and 66, respectively (Fig. 5). Clones from introduced populations were distributed between two of these clusters (1, 3). The first branch of the tree was composed largely of clones identified from introduced populations, except for two genotypes (W and Br.Cl) that were found in both introduced and native populations. The second branch was

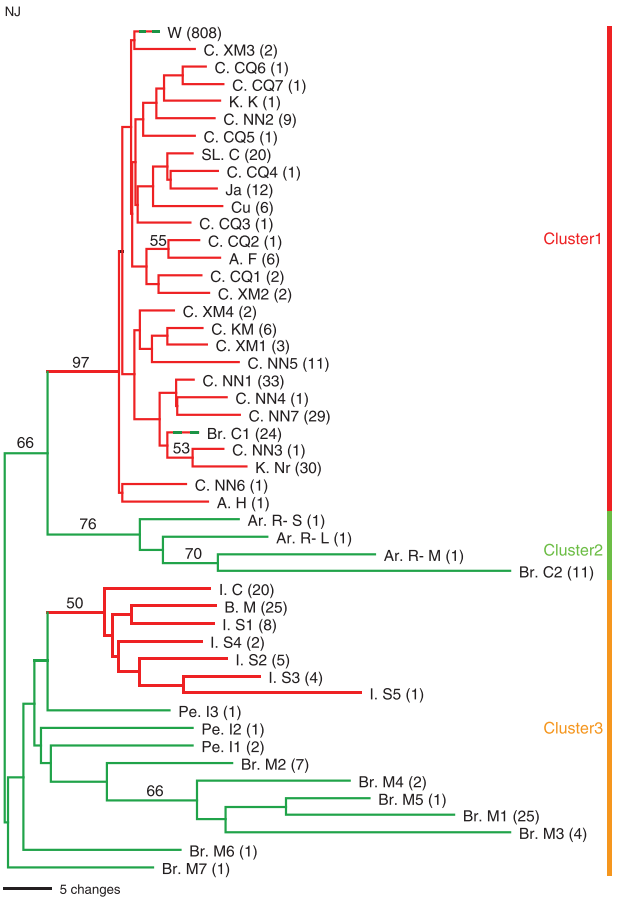

Fig. 5 Neighbour-joining tree of 49 clones of *Eichhornia crassipes* constructed using simple mismatch distances rooted by midpoint. Red vs. green branches indicate genotypes sampled from the introduced and native range, respectively. Red and green branches represent genotypes found in both the native and invasive range. Bootstrap values (>50) are shown above the branches. Two genotypes (W and Br.C1) occurred in both introduced and native populations. The 49 clones are grouped into three main clusters with genotypes found in introduced populations clustered into two different branches. Numbers in brackets indicate the number of samples assigned to that genotype.

composed of four clones from the native range in Argentina and southern Brazil. The third branch was composed of clones from Amazonia (Manaus, Brazil and Iquitos, Peru) and from Bangladesh and Indonesia. A striking feature of the dendrogram of clonal relationships is that the genetic distances among introduced populations were small, as indicated by the short branch lengths (see cluster 1), whereas branch lengths among native populations were usually much larger. The exception to this pattern is the large branch lengths of clones from the distinct cluster of introduced populations from Bangladesh and Indonesia. PCoA analysis revealed similar results to the neighbour-joining tree

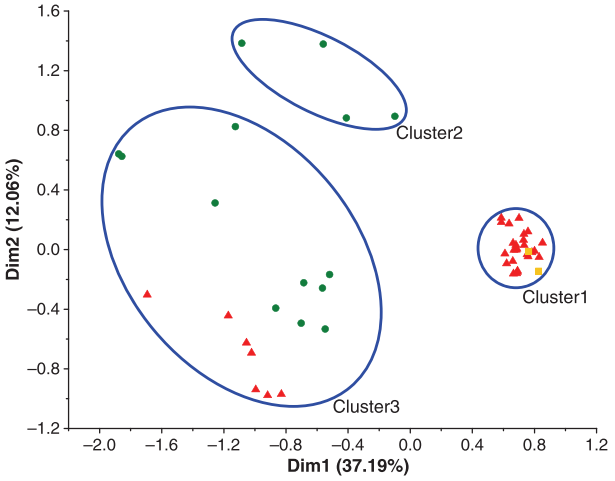

Fig. 6 Principal co-ordinate analysis plot for the first two principle co-ordinates of pairwise genetic distances among 49 clones of *Eichhornia crassipes*. Red triangles indicate genotypes found exclusively in invasive populations, green circles are found exclusively in native populations, and orange squares represent genotypes found in both native and introduced population. Blue circles circumscribe genotypes corresponding to the three clusters in the neighbour-joining tree.

(Fig. 6). The first and second axis explained 37.2% and 12.1% of the total variation, respectively.

Discussion

The main finding of our global survey of genetic diversity in water hyacinth (*E. crassipes*) is the low levels of variation that occur throughout the introduced range. Eighty percent of adventive populations were genetically uniform and a widespread clone ('W') detected in two Peruvian populations accounted for 75.9% of the individuals sampled from the invasive range (Table 1). Genetic uniformity likely results from successive nested bottlenecks during range expansion, predominant clonal growth, and limited opportunities for sexual recruitment at most sites. We discuss below how these features have likely influenced the geographical patterns of clonal diversity and consider ecological and genetic hypotheses contributing to the success of *E. crassipes* as an invasive species.

Low diversity characterizes introduced populations

Although only a small number of native populations ($n = 7$) were surveyed in our study compared to introduced populations ($n = 47$), we found a significant reduction in genetic variation associated with migration to the adventive range. This finding is consistent with geographical surveys of style morph distribution (Barrett & Forno 1982; Barrett 1989), and two previous

surveys of genetic diversity in introduced populations from China (Ren *et al.* 2005; Li *et al.* 2006). However, our results indicate that introduced populations do not always rely solely on clonal propagation, as previously proposed (Li *et al.* 2006), although sexual reproduction probably occurs less often than has been suggested by Ren *et al.* (2005).

Our cluster analysis of the genetic relationships of clones (Fig. 5) indicates that the introduction of *E. crassipes* to its adventive range has involved a very limited sampling of clonal diversity from South America. Although the number of introductions cannot be determined with certainty, it seems likely that relatively few were involved. Documented records suggest that *E. crassipes* has experienced repeated nested bottlenecks associated with local dispersal during range expansion. For example, in eastern Asia the species was introduced in the 1930s to Taiwan and mainland China, probably from Japan (Ding *et al.* 2000). All introduced genotypes, except some from Bangladesh and Indonesia, clustered into one branch, including the predominant genotype 'W' (Fig. 5). Some clones in Bangladesh and Indonesia appear to have a different origin from those in cluster 1, as they contain genotypes that form a separate branch of the dendrogram (cluster 3) also containing several native genotypes (Figs. 5 and 6). Significantly, the widespread clone 'W' was also found in the upper Amazon at Iquitos, Peru raising the possibility that this area could have been one of the primary sources for the worldwide invasion of *E. crassipes*. However, without more extensive surveys in South America, particularly from the Orinoco River in Venezuela, which historical records implicate as an early source region (Sculthorpe 1967), this conclusion would be premature. At this stage it is not possible to locate the likely source regions, or say with any confidence how many separate introductions were involved in colonization of the introduced range. If further sampling reveals that clone 'W' has a wider distribution in the native range than our survey indicates, it would raise the possibility that multiple introductions of this clone to the adventive range could have occurred. However, because of the greater opportunities for sexual reproduction in the native range we consider this possibility unlikely.

Clonal propagation predominates but with occasional sex

Our study revealed that ~80% of introduced populations of *E. crassipes* were composed of a single clone. This demonstrates that clonal propagation is the predominant mode of reproduction in the introduced range. Floating propagules of *E. crassipes* invade previously unoccupied sites and with subsequent dispersal to additional sites large areas of genetic uniformity are created. However, it is important to emphasize that this process is not restricted to the introduced range of *E. crassipes*, as many populations occurring in the native range are composed of a single floral morph and some are likely to be single clones (Table 1 in Barrett & Forno 1982). Nevertheless, the incidence of multiclonal populations is much higher in the native range, where outcrossing among the three floral morphs is promoted by specialized long-tongued bees, and environmental conditions favouring sexual recruitment are more frequently encountered (Barrett 1977). More extensive sampling of populations in South America will almost certainly reveal higher levels of genetic diversity than were detected in our study.

Earlier workers concluded that introduced populations of *E. crassipes* were largely clonal because they were incapable of reproducing sexually (reviewed in Barrett 1980a; b). This conclusion was based on three mutually exclusive assumptions: (i) because the species is tristylous, clones are self-incompatible and therefore seed cannot be produced in populations containing a single floral morph; (ii) some clones are genetically sterile because of a long history of vegetative reproduction and limited selection against the build up of sterility mutations; (iii) a paucity or absence of insect visitors to *E. crassipes* in the introduced range results in little seed set. However, subsequent work has shown that each of these assumptions is false. Clones of *E. crassipes* are not genetically sterile and are capable of producing abundant seed from cross- or self-pollination (Barrett 1980*a*). Although levels of seed set in the introduced range are lower than the native range, pollinators visit many populations and seed is commonly produced (Barrett 1980*b*). Sexual reproduction in most *E. crassipes* populations is prevented because of unfavourable environmental conditions for seed germination and seedling establishment. High temperatures (>30 °C) and exposed wet mud associated with water level fluctuations promote seed germination and establishment. These conditions are absent in many introduced habitats, where seeds either sink into deep water, or are shaded by the dense vegetative mats created by vigorous clonal propagation. Thus, ecological rather than genetic factors limit sexual reproduction in most introduced populations of *E. crassipes*.

Not all adventive populations of *E. crassipes* we surveyed were composed of single clones. We detected genetic diversity in several populations (Table 1), although in each case co-occurring clones belonged to the same genetic cluster and branch lengths were much shorter than were observed in most native populations (Fig. 5). This suggests that the genetic diversity within introduced populations has arisen primarily from inbreeding as opposed to multiple introductions of

different clones. Inbreeding is promoted in adventive populations because large clone sizes and self-compatibility promote geitonogamous self-pollination when pollinators visit populations. However, not all selfing in *E. crassipes* arises from pollinator-mediated geitonogamy. Mating-system modifiers of the M-morph causing autonomous self-pollination are reported from several *E. crassipes* populations that we surveyed from China that contained clonal diversity (Chongqing, Kunming, and Nanning populations, see Ren *et al.* 2005). Furthermore, the Chongqing population in which conditions suitable for sexual reproduction occur (*unpublished observations*), was composed of both the L- and M-morphs. This suggests that the L-morph may have arisen from selfing and genetic segregation, as reported for a population from Costa Rica also containing selfing variants of the M-morph (Barrett 1979). Stylar dimorphism can originate if genotypes present in a population are heterozygous (*Mm*) at the style length loci because the L-morph is the recessive genotype (*mm*). The sporadic occurrence of the L-morph in populations throughout the introduced range can serve as a signature of local episodes of sexual reproduction in populations containing heterozygous genotypes of the M-morph.

Ecology and genetics of invasions

The success of invasive species can depend on their ability to adapt to local environmental conditions (Sakai *et al.* 2001; Maron *et al.* 2004; Bossdorf *et al.* 2005; Montague *et al.* 2008; Prentis *et al.* 2008; Colautti *et al.* 2009). Evolution of local adaptation will be promoted by standing genetic variation for ecologically relevant traits (Colautti *et al.* 2010), as well as new mutations. Multiple introductions, genetic admixture and the absence of repeated bottlenecks all aid in maintaining diversity (Colautti *et al.* 2006; Roman & Darling 2007; Dlugosch & Parker 2008). However, our results suggest that the contemporary evolution of local adaptation is unlikely to have played a prominent role in the worldwide spread of *E. crassipes.* Most invasive populations contain very little if any genetic variation and opportunities for genetic recombination are restricted by unfavourable environmental conditions. Rather, *E. crassipes* probably owes its invasive success to the possession of several key life-history traits, including the high dispersal of floating propagules, prolific clonal reproduction and well-developed phenotypic plasticity. These traits are adaptive for the ecological conditions that *E. crassipes* encounters in its native range where dramatic water level fluctuations characterize riverine, lake and wetland habitats. These traits also enhance the ability of *E. crassipes* to colonize and proliferate in its introduced range.

To our knowledge this study represents the first global survey of genetic diversity in a highly clonal invasive flowering plant species. Elsewhere, surveys of genetic diversity in clonal plants have revealed considerable variation in patterns of diversity within and among species, but no grounds for assuming that clonal plant populations are commonly genetically depauperate (reviewed in Ellstrand & Roose 1987; Silvertown 2008). In contrast, studies of invasive clonal aquatics have often revealed very low levels of diversity in the introduced range as a result of founder effects and restrictions on sexual reproduction (e.g. Scribailo *et al.* 1984; Barrett *et al.* 1993; Hollingsworth & Bailey 2000; Kliber & Eckert 2005; Wang *et al.* 2005). However, it is important to recognize that low levels of diversity at marker loci in invasive plants are not only associated with predominant asexual reproduction. There are numerous examples of highly selfing species in which genetic bottlenecks have resulted in low diversity in the introduced range in comparison with the native range (reviewed in Barrett & Shore 1989; Novak & Mack 2005; Barrett *in press*). However, unlike the clonal populations investigated in this study, many predominantly selfing populations contain considerable amount of quantitative genetic variation for ecologically-relevant traits enabling the evolution of local adaptation.

Our findings highlight the important role of stochastic processes in determining the geographical patterns of genetic diversity in invasive species. However, it is possible that the prevalence of the 'W' genotype throughout the invasive range is not entirely a result of chance sampling effects. This particular clone may exhibit superior physiological and life history attributes compared to other clones. These features could have enabled it to colonize a wide range of environments in essence behaving as a 'general purpose genotype' with high phenotypic plasticity (Baker 1965; Richards *et al.* 2006; Hulme 2008). In the native range, clone 'W' was sampled from mountainous regions of Peru (Puerto Maldonado), as well as in the Upper Amazon Basin near Iquitos (Peru). This raises the possibility that clone 'W' may be able to proliferate over a broad range of environmental conditions, including those with relatively low temperatures. In the introduced range this clone was the only genotype found in Europe and it also occurred commonly in China, both regions prone to low temperature and occasional winter frosts. Future studies comparing the performance of clones, including 'W', sampled from diverse locations in the native and introduced range across a range of environmental conditions would be valuable for testing this hypothesis. Regardless of the outcome of such experimental comparisons, our study demonstrates that significant amounts of genetic diversity are clearly not always a

pre-requisite for global invasive success over contemporary time scales in invasive plants.

Acknowledgements

We thank the many colleagues too numerous to list here for assistance with sampling clones of water hyacinth and Sarah Yakimowski and Stephen Wright for providing comments on the manuscript. This work was financially supported by the State Key Basic Research and Development Plan (2009CB119201), the National Natural Science Foundation of China (30430160), the Cultivation Fund of the Key Scientific and Technical Innovation Project from Ministry of Education of China (704005) to D-YZ and a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada to SCHB.

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