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Genetic diversity and clonal vs. sexual reproduction in *Fallopia* spp. (Polygonaceae)[1]

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Although fundamental to the study of invasion mechanisms, the relationship between mode of reproduction and plant invasion is not well understood. *Fallopia japonica* (Japanese knotweed), a highly aggressive invasive plant in both Europe and North America, serves as a model species for examining this relationship. In Britain, *F. japonica* var. *japonica* is a single female clone reproducing solely through vegetative growth or obligate hybridization with other *Fallopia* spp. In the U.S., however, there is more evidence for sexual reproduction. Here, simple sequence repeat (SSR) markers were developed, and three Massachusetts populations were sampled at regular intervals. The amount of sexual and clonal reproduction in each population was determined based on within-population genetic diversity. Clonal growth was apparent, but the populations together contained 26 genotypes and had evidence of sexual reproduction. One genotype that was present in all populations matched the single aggressive British clone of *F. japonica* var. *japonica*. Also, a potentially diagnostic marker for the *F. sachalinensis* genome provided evidence of inter- and intraspecific sexual reproduction and introgression. These differences observed in U.S. populations compared to European populations have significant implications for management of *Fallopia* spp. in the U.S. and underscore the importance of regional studies of invasive species.

Key words: clonal reproduction; *Fallopia japonica*; genetic diversity; Japanese knotweed; microsatellite; *Polygonum cuspidatum; Reynoutria japonica*; sexual reproduction.

Invasive species are a persistent threat to native biodiversity and functioning ecosystems and extract significant ecological and economic costs (Vitousek et al., 1996; Mack et al., 2000; Olden et al., 2004; Pimentel et al., 2000). Many life history traits such as self fertilization and vegetative reproduction appear associated with "weediness" in invasive plant species (Stebbins, 1957; Baker, 1974; Rejmánek, 2000; Kolar and Lodge, 2001), and several studies have provided evidence for rapid shifts in reproductive traits associated with invasions (Brown and Marshall, 1981; Daehler et al., 1999; Leiss and Muller-Scharer, 2001; Jakobs et al., 2004; Brown and Eckert, 2005). While the drivers of these changes are often likely complex, they may sometimes prove to be as simple as the addition of an efficient pollinator (Richardson et al., 2000 and references therein). As Sakai et al. (2001, p. 306) stated, "Invasions are like natural experiments …", and species that have invaded multiple regions of the world provide the replicates of these natural experiments. A comparison of invasion trajectories for such a species will allow a dissection of the dynamics and role of reproductive shifts during the lag time between the introduction and the expansion of an invader. *Fallopia japonica* (Houtt.) Ronse Decr. (*Polygonum cuspidatum* Siebold & Zucc., Japanese knotweed) appears to be an excellent model system for these kinds of studies.

The herbaceous perennial *F. japonica* is a highly aggressive, invasive species in both Europe and North America. It ranks among the world's 100 worst invasive alien species (Global Invasive Species Database, 2005), is a noxious weed in four U.S. states, and is banned, quarantined, or prohibited in four others (USDA, NRCS, 2006). A native of China, Japan, and Korea, it was introduced to the UK as a garden ornamental sometime after 1830 and was reported as naturalized within the U.S. by 1894 (Merhoff et al., 2003). Herbarium records indicate the presence of *F. japonica* in Massachusetts as early as 1877 (Forman and Kesseli, 2003), and a recent study has documented its expansion from the northeast U.S. to nearly every state and province in the U.S. and Canada, respectively (Barney, 2006). This species was likely introduced to the U.S. from Europe, but direct introductions from Asia also seem likely (M. Gammon, unpublished data). Most studies examining populations of Japanese knotweed have taken place in Europe or Asia, and until recently (Forman and Kesseli, 2003), it was assumed that U.S. populations were similar to European populations in their structure and mode of reproduction.

Clonal spread by rhizomes and rapid growth, resulting in vast monocultures that outcompete native vegetation, have been considered the major features making *F. japonica* a problematic invader that is difficult to control. The stems are hollow and bamboo-like, forming dense stands up to 3 m high, and the understory growth of other species is restricted by the accumulation of leaf and stem litter as well as a canopy that blocks light (Beerling et al., 1994). Tiny pieces of rhizome or stem are capable of regrowth and have been considered important dispersal propagules (Beerling et al., 1994; De Waal, 2001). Physical removal of knotweed may be a realistic management solution only for small populations (Soll, 2004) and has even been speculated to promote its spread (Beerling and Palmer, 1994). Various herbicide treatments are effective, but multiple applications may be necessary and impacts on nontarget organisms as well as surrounding waterways must be considered (Soll, 2004). Biological control methods, which use host-specific pathogens or insects to control invasives, are also under investigation for Japanese knotweed (Shaw and Seiger, 2002), but no control strategy has proven to be the single best approach.

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The breeding system of Japanese knotweed has been characterized as dioecious and gynodioecious in Britain, but no male-fertile plants of *F. j.* var. *japonica* have been observed (Bailey, 1994; Beerling et al., 1994). All *F. j.* var. *japonica* plants are female (Bailey, 1994) and based on genetic evidence, likely belong to a single clone (Hollingsworth and Bailey, 2000) presumably dispersed as rhizome fragments. Seeds are considered rare, and all seeds observed on these plants are reported to be the product of hybridization with *F. sachalinensis* (F. Schmidt) Ronse Decr. (*Polygonum sachalinense* F. Schmidt; giant knotweed) or *F. baldschuanica* (Regel) Holub (*Polygonum baldschuanicum* Regel, Russian vine, silver lace vine) (Bailey, 1994). Sexual reproduction by hybridization has recently been documented as contributing to the success of Japanese knotweed in Belgium (Tiebre et al., 2007) and in the Czech Republic, where *F. ×bohemica* appears to spread faster than either parental species (Mandák et al., 2004). As hybridization may be a significant factor in invasion biology of many species (Ellstrand and Schierenbeck, 2000), and aggressive hybrids have been documented for other species such as British cordgrass, *Spartina anglica* (Thompson, 1991) and johnsongrass (Paterson et al., 1995), the structure of European populations of *F. japonica* may be changing.

Contrary to what has been reported in Britain, in the U.S., *F. japonica* is capable of sexual reproduction. In North America, *F. japonica* has been characterized as having a "leaky" dioecious or subdioecious system, with populations of both male and female plants that still maintain vestigial reproductive parts of the other sex. Some male ramets do set a few seeds (up to 100) but female plants producing pollen have not been observed (Forman and Kesseli, 2003). Seeds on female ramets are extremely abundant, with tens of thousands produced each year, and these can germinate under a variety of conditions (Forman and Kesseli, 2003; Bram and McNair, 2004). Although sexual reproduction of this species in the U.S. has been verified, very little is known about the overall contribution of sexual reproduction to the spread of this species or the origin of seeds. Furthermore, even though methods for identifying hybrid *F. ×bohemica* have been devised (Zika and Jacobson, 2003), the actual degree of hybridization in the U.S. is still not known, mostly because accurate identification of hybrids using morphological traits is difficult (Gammon et al., 2007). In Massachusetts, *F. japonica* is far more widespread than *F. sachalinensis* (Forman, 2003; Gammon et al., 2007), and although there is now evidence for first and later generation hybrids, the level and scale of introgression are still unclear (Gammon et al. 2007).

To date there have been no genetic studies demonstrating the extent of genetic diversity within U.S. populations or the role of sexual reproduction in dispersal of this species. In the present study we characterized, with simple sequence repeat markers (SSRs), also known as microsatellites, the level of genetic diversity of three populations of Japanese knotweed. Based on within-population genetic diversity, we determined the degree of clonal and sexual reproduction within each. Moderate levels of gene diversity were detected, suggesting that the populations are the product of both sexual and clonal reproduction. This study clarifies the population dynamics of this species in the northeastern U.S. and will contribute to the development of more effective management strategies. Differences observed in U.S. populations compared to those described in Europe underscore the importance of regional studies when studying invasive species and devising plans for management.

MATERIALS AND METHODS

SSR detection—The SSRs were detected using the approach of Toonen (1997). Single-stranded oligonucleotides composed of di- and trinucleotide repeats were labeled using a nonradioactive digoxygenin (DIG) oligo 3′ end labeling kit (Roche, Basel, Switzerland). Genomic DNA of a suspected hybrid, *F. ×bohemica*, (collected from Walpole, Massachusetts, USA, classified based on morphology) was extracted using a DNeasy Plant Maxi extraction kit according to recommended protocol (Qiagen, Valencia, California, USA), then precipitated with ethanol. Genomic DNA was digested overnight with *Mbo*I, size-selected (300–900 bp), and excised from the gel. DNA was purified using a Qiaex II kit (Qiagen) and ethanol-precipitated. This DNA was then ligated into a linearized and dephosphorylated pBlue-script II KS(+) phagemid vector (Stratagene, La Jolla, California, USA). Vectors containing *F. japonica* inserts were then transformed into competent *E. coli* cells (JM109, Promega, Madison, Wisconsin, USA), and cells were plated onto LB agar plates amended with ampicillin, IPTG, and X-gal. Selection of DNA for appropriate insert size was verified by PCR with M13 universal primers.

All PCRs were done in 25 μL with 1 μL of diluted DNA (approximately 20–100 ng), 1 μL of each primer (10 pmol/μL), 2.5 μL of 10× reaction buffer, 2.5 μL of 2.5 mM combined dNTPs, 2.5 μL of 25 mM $MgCl_2$, 2.5 μL of 100× BSA, and 0.2 μL of *Taq* polymerase. The remaining volume was brought up to 25 μL with water. Amplification was performed with the following cycles: 5 min denaturation at 94°C; 35 cycles of 94°C for 30 s, 54–60°C for 30 s, 71°C for 30 s; followed by a final extension at 71°C for 5 min.

White colonies were replated onto grid-plates, grown again at 37°C overnight, and lifted onto Hybond-N+ DNA transfer membranes (GE Healthcare, Buckinghamshire, UK). Lifted colonies were again grown on agar plates for 2–3 h, and membranes were prepared, hybridized with a DIG-labeled dinucleotide repeat probe (TC_{10}), and washed as described by R. Toonen (1997). A DIG-antibody was applied to the membranes, and a DIG nucleic acid color-detection system (Roche) was used to detect this antibody. Positive colonies were grown in 2XYT buffer, and plasmid DNA was isolated using a plasmid mini-prep kit (Qiagen). Plasmid DNA was amplified by PCR using M13 primers. PCR fragments underwent a cycle-sequencing reaction using dye-terminated dNTPs. Fragments were sequenced on a 3100 Avant sequencer (ABI, Foster City, California, USA) to confirm the presence of SSRs.

If a SSR was confirmed by sequencing, primers flanking the repeat were designed with the PRIMER3 program (Rozen and Skaletsky, 2000) and conditions were optimized for PCR. Next, a forward or reverse primer labeled with a fluorescent tag was used for PCR for fragment analysis.

DNA fingerprinting of populations—Three populations (Alewife in Cambridge, Massachusetts (MA); Braintree, MA; and Neponset River in Boston, MA) of *F. japonica* sensu lato and one control sample from Corley, Warwickshire, UK (donated by J. P. Bailey) were selected for analysis during the summer 2005. There were no stands or individuals that would be defined as *F. sachalinensis* in the area, but the distinction between pure *F. japonica* and *F. ×bohemica* is not clear (Gammon et al., 2007). The populations were all generally linear, terrestrial, along pedestrian pathways, and in disturbed habitats. All populations contained both male and female patches (scored during flowering), suggesting initially that there were *at least* two genets within each of these populations. In contiguous stands, individual ramets were sampled along transects at regular intervals (5 m in Neponset and Alewife and 2 m in the smaller Braintree site). Small isolated patches along the transects were also sampled.

Leaves were stored in DriRite drying agent crystals (W.A. Hammond Drierite Co. Ltd, Xenia, OH, USA) until DNA extraction. DNeasy Plant Mini extraction kits (Qiagen) were used for DNA extractions following manufacturer's protocols. All DNA samples were scored for the SSR markers developed in this study (Table 1), as well as one, PCU1, previously developed by Miwa et al. (2001). Fragments were amplified with a fluorescent primer designed for each SSR (except KW6), and products were diluted 1 : 20, mixed with Hi-Di formamide (ABI) and assayed on the 3100 Avant sequencer (ABI). Gene Mapper software was used for analysis (ABI). Peaks were assigned numbers by Gene Mapper, which approximated the length of the amplicon. To aid interpretations of complex patterns, some samples were also loaded onto a 6% denaturing acrylamide gel containing urea and stained with silver sequence

TABLE 1. Descriptions and amplification conditions of simple sequence repeat markers developed from *Fallopia* ×*bohemica*.

Locus name (GenBank accession)	Repeat motif[a]	Primer sequences 5′ → 3′	Fluorescent label	Size range (bp)[b]	Size of cloned fragment (bp)[a]	T_a (°C)	No. of alleles/ locus (null)
KW2 (EF088498)	($GA^{34}NN \ldots GA^{17}$)	F: CGATGGAGTAGGTCTTATCTATTTAT R: CCTCTACTCAGTTCTCTAGTGAAGGTC	6-FAM	456	ND	54	2 (1)
KW3 (EF088499)	$(TC)_{19}$	F: CTCCGTCAATTCTCCCACTC R: AAAAGCGGATGGTTGACAAA	6-FAM	132–178	178	56	2
KW4 (EF088500)	Compound TC repeat	F: TCCATGCTCCTAAAGTGCAA R: TCTCCGTTTTGAGGTGGTTC	HEX	200	233	56	2 (1)
KW5 (EF088501)	$(TC)_{28}$	F: TTCGTTCCGTAGTTGCCATA R: GCAGGGAAGGAAAAGAAGGT	NED	187–219	220	60	5
KW6 (EF088502)	GAGGG$(AG)_5$ AAA$(GA)_6$	F: TGGTTTTGTTTCAAGTTTCTTGTG R: TGTTGATGGTTGGTTGCTTC	no label	338	338	54	2 (1)
KW7 (EF088503)	$(CT)_{16}$	F: GAGGTTTGCTCTCTCT R: CACTTTTCTCTTTGACTCCGATA	6-FAM	207–215	206	56	2
PCU1a[c], PCU1b[c] (AB055129)	$(AT)^2(GT)^{35}$	F: ACGTAAAACACATATATGCAGTG R: TCATGTCTTACGCATACAATTAC	HEX	101–112 146–150	ND	50[d]	4 (1) 2

[a] Number of nucleotide repeats and size of cloned fragment were approximated because sequencing through some repeats was difficult.
[b] Table only includes fragments used for genotype analysis.
[c] PCU1 marker revealed two polymorphic loci and was developed by Miwa et al. (2001).
[d] Annealing temperature adjusted to 50°C.
ND = No data.

staining reagents (Promega). Some primer pairs produced additional peaks that were either not polymorphic or were interpreted as separate loci, and these were ignored (Table 1).

Allele frequencies and statistical analyses—Because *F. japonica* and related taxa are polyploids with $2n = 44$, 66, 88, 102, 110, and even 132 (Mandák et al., 2003 and references therein), genotyping results were sometimes complex. In Massachusetts, flow cytometry data suggested ploidies of $2n = 44$, 66, 88, and in one case, 54 (Forman, 2003). We assumed a standard Mendelian locus at all proposed loci used for genotyping because there were no more than two alleles/individual. Given that several loci contained null alleles, F_{ST} values were calculated with the C2 correction of FreeNA (Chapuis and Estoup, 2007). Expected and observed heterozygosity for determining the inbreeding coefficient (F_{IS}) were calculated using Genepop (http://wbiomed.curtin.edu.au/genepop/) for codominant loci only (Raymond and Rousset, 1995).

The clonal diversity was estimated using a modified Simpson's diversity index approach (Pappert et al., 2000; Dong et al., 2006); $D = 1 - \Sigma\, n_i(n_i - 1)/[N(N - 1)]$, where N is the total number of samples, and n_i is the number of samples of the ith genotype present in that population. The index D ranged from 0 to 1; 0 indicated an entirely clonal population, and 1 indicated a population composed only of unique genotypes and no clones.

To avoid sampling effects, we calculated the average number of ramets per square meter along the transects of each population. We incorporated this average into the Simpson's diversity index calculation to better estimate the number of individuals for a given genotype and to ensure that the amount of asexual reproduction was not underestimated. At five sampling points per population, a 1-m^2 quadrat was placed on the ground with the sampled shoot at the center of the quadrat. We assumed that shoots within the quadrat surrounding the core group were all ramets of one genet, and this could be verified in some cases because shoots were clearly connected by rhizomes. The numbers of ramets per square meter were then averaged for each population and used to estimate the number of ramets per clone. If adjacent samples along the transect were scored as the identical multilocus genotype, we assumed that the genotype covered the entire span between sampling points. If adjacent samples were different genotypes, we assumed that the genotype changed midway between sampling points. If there were discrete gaps between samples, the area covered by the clone was estimated with a minimum area of 1 m^2.

RESULTS

SSR screen and population diversity—We screened 1891 kb of *F.* ×*bohemica* genomic DNA for SSRs (3782 colonies each containing an average of 500 bp of inserted DNA). Six polymorphic SSR loci were discovered in this study (Table 1), all of which contained either a simple or compound CT/GA dinucleotide repeat. These six SSRs detected two to five alleles per locus. Several primer pairs in this study amplified multiple fragments, but the peaks used for the analyses were polymorphic, reproducible, and informative for genotyping. PCU1 from Miwa et al. (2001) amplified fragments within the range reported. In calculating allele frequencies for PCU1, two separate loci were determined, one ranging from 101–112 bp with an additional null allele, and one from 146–150 bp. Microsatellites are typically codominant, but four of eight of these loci had null alleles (KW2, KW4, KW6, and PCU1a). This may be a characteristic of polyploid genomes, particularly those derived from interspecific hybridization (Roder et al., 1995; Saltonstall, 2003).

Expected heterozygosity was moderate for each population, ranging from 0.317 to 0.430 (Table 2). There was no evidence of inbreeding within populations because F_{IS} values for the codominant loci of each population were not consistently positive; means for the populations ranged from −0.313 to 0.096 (Table 2). Interestingly, the Neponset population with the highest gene diversity (expected heterozygosity) and the most multilocus genotypes appeared to have a consistent excess of heterozygotes (negative F_{IS}). Also, minor population substructure was detected; pairwise F_{ST} among populations ranged from 0.084 to 0.198. The global F_{ST} was 0.124 (95% CI: 0.040–0.241). The two populations (Braintree and Alewife) with the lowest diversity and fewest genotypes were most diverged ($F_{ST} = 0.198$), suggesting that genetic drift because of small founding populations may be important. These two populations were also the most geographically distant.

The total number of alleles per locus was not extremely high for microsatellites, suggesting few genets per population and even fewer individuals in each of the original founder populations. There were five to 15 distinct multilocus genotypes (genets) for the 21 to 23 samples of each population. All the multilocus genotypes per population could be generated by recombination from a minimum of two to four founders in each. Only two would be needed in Alewife (female B × male E) and Braintree (female B × male K or female H or I × male

TABLE 2. Expected heterozygosities (H_e), observed heterozygosities (H_o)[a], inbreeding coefficients (F_{IS})[a], F_{ST}, and pairwise F_{ST} for three populations of Japanese knotweed.

Locus	Alewife			Braintree			Neponset			All populations	
	H_e	H_o	F_{IS}	H_e	H_o	F_{IS}	H_e	H_o	F_{IS}	F_{ST}	F_{IS}
KW2	0.002	NA	NA	0.401	NA	NA	0.421	NA	NA	0.472	NA
KW3	0.136	0.143	−0.053	0	0	—	0	0	—	0.051	−0.054
KW4	0.002	NA	NA	0.002	NA	NA	0.466	NA	NA	0.351	NA
KW5	0.486	0.381	0.216	0.419	0.217	0.481	0.631	0.727	−0.153	0.014	0.140
KW6	0.452	NA	NA	0.468	NA	NA	0.356	NA	NA	0.003	NA
KW7	0.486	0.381	0.216	0.488	0.826	−0.692	0.506	0.682	−0.346	0.026	−0.290
PCU1a	0.662	NA	NA	0.566	NA	NA	0.567	NA	NA	0.090	NA
PCU1b	0.314	0.381	−0.212	0.470	0.739	−0.571	0.489	0.727	−0.487	0.176	−0.455
All loci	0.317 ± 0.231	0.321	0.096	0.352 ± 0.208	0.446	−0.294	0.430 ± 0.180	0.534	−0.313	0.124	

Note: F_{ST} (Alewife-Braintree) = 0.198; F_{ST} (Braintree-Neponset) = 0.084; F_{ST} (Alewife-Neponset) = 0.088.
[a] H_o and F_{IS} could not be calculated for loci with dominant alleles.

G), while four founders (female B × males P, S, N; or female O × male P, S or V, N) would be required in Neponset. Surprisingly, the only genet (genotype B) observed in more than one population and the only one that could be a founder in all populations was a female, and it was identical to the aggressive clone found throughout Britain (Table 3).

Sexual and clonal reproduction—Based on the adjusted Simpson's diversity index that integrates ramet density into the equation, all populations appear to have been derived from a mixture of sexual and asexual reproduction. We identified 26 genets, defined as multilocus genotypes, in the 66 different samples. The Simpson's diversity index values did not indicate purely sexual ($D = 1.0$) or asexual ($D = 0$) reproduction for any population (Table 3). Populations possessed both small genets, composed of as few as four ramets all within 1 m^2, and massive genets likely covering more than 100 m^2. Alewife, which contained the fewest genets (four males and a single female) had a value of $D = 0.58$. One male genet (genotype A) was sampled multiple times over a contiguous track that spanned 45 m, while the three remaining males were restricted and detected only once or twice. The one female genet (genotype B) formed large patches scattered throughout the population. This could represent one contiguous clone recently subdivided by bike path construction and other activities, but this could not be determined. Braintree was similar to Alewife in level of diversity ($D = 0.62$) and, like Alewife, had a mixture of large and small genets. The 23 samples represented eight genets (three males and five females). The largest genet (male genotype G) was sampled 12 times and appeared to span 28 m, while the smallest genets were sampled only once and appeared restricted to 1 m^2. The Neponset population was the most diverse ($D = 0.73$) of the three populations. There were 15 genets (five females and 10 males) among the 22 samples (Table 3). The population possessed several small genets covering less than 1 m^2 as well as one extensive clone (genotype B) appearing to extend more than 60 m.

In calculating Simpson's diversity index, we also separated males from females in the populations to examine differences in their reproductive strategies (Table 3). There was no consistent trend in all three populations. In Alewife, female samples were only the product of asexual reproduction ($D = 0$) because they were all identical and represented only one genet. Male ramets also often appeared to be the result of asexual reproduction, but some were likely produced from sexual reproduction ($D = 0.32$). Although the Braintree population had a very similar overall diversity index to Alewife, when males and females were considered separately, Braintree females were largely the product of sexual reproduction ($D = 0.74$), while males were mostly clones ($D = 0.15$). In the Neponset population, males were mostly the product of sexual reproduction ($D = 0.84$), while females were generally from clonal growth.

The dynamics of establishing populations, vs. expanding them, appear starkly different. Patches initiated by seed dispersal would be characterized by unique genets because sexual reproduction would result in new allele combinations and wind would disperse the seed some distance away. Alternatively, if separate patches were founded by dispersed pieces of rhizomes, they should be composed most often of genets from neighboring patches. Using the presence of unique genotypes within a physically separate stand as an indicator of seed dispersal, we found that 17 of 21 separate stands sampled had at least one genotype unique to that stand. This was particularly evident when examining the 10, small (<1 m^2), isolated patches

TABLE 3. Clonal diversity of three populations of Japanese knotweed.

Population	N	Multilocus genotypes	D_{total}	N^a	D_{total}^b	D_{female}^b	D_{male}^b
Alewife	21	**A(m,9)**, B(f,8), C(m,1), **D(m,1)**, **E(m,2)**	0.69	1943	0.58	0	0.32
Braintree	23	B(f,3), **F(m,1)**, **G(m,12)**, H (f,3), I(f,1), J(f,1), **K(m,1)**, L(f,1)	0.72	1123	0.62	0.74	0.15
Neponset	22	B(f,8), **M(m,1)**, **N(m,1)**, O(f,1), **P(m,1)**, **Q(m,1),** R(f,1), S(m,1), **T(m,1)**, **U(m,1)**, **V(m,1)**, W(f,1), X(m,1), **Y(f,1)**, **Z(m,1)**	0.88	673	0.73	0.42	0.84

Note: Multilocus genotypes represented by letters (sex, number of ramets sampled with that genotype, hybrids based on KW6 marker are in bold); N = number of ramets sampled; D = modified Simpson's diversity index, $D = 1 - \Sigma\, n_i(n_i - 1)/[N(N - 1)]$.
[a] Estimated number of ramets in the transect of the population.
[b] n_i and N were adjusted to represent the estimated number of ramets per clone and estimated number of ramets in the transect of the population, respectively.

TABLE 4. KW6 amplification in samples from the UK, Washington (WA), USA, and Massachusetts (MA), USA.

Sample ID	Taxon (number of samples)	Origin of sample	KW6 (±)	Classifier
80	*Fallopia japonica* var. *japonica* (1)	UK	−	J. Bailey
53	*Fallopia japonica* var. *japonica* (1)	WA	−	P. Zika, J. Forman Orth
Multiple IDs	*Fallopia japonica* var. *japonica* (17)	Various MA sites	±[a]	M. Gammon
UK Fabo	*Fallopia* ×*bohemica* (1)	UK	+	J. Bailey
50, 51, 54, 55–57	*Fallopia* ×*bohemica* (6)	WA	+	P. Zika, J. Forman Orth
52	*Fallopia sachalinensis* (1)	WA	+	P. Zika, J. Forman Orth
82	*Fallopia sachalinensis* (1)	Weston, MA	+	J. Forman-Orth
84	*Fallopia sachalinensis* (1)	Scituate, MA	+	M. Gammon
141	*Fallopia sachalinensis* (1)	Peru, MA	+	M. Gammon
172, 173	*Fallopia sachalinensis* (2)	Nantucket, MA	+	J. Forman Orth

[a] Fifteen of 17 samples did not produce the fragment; the two samples with the allele now appear to be the progeny of hybrids that have backcrossed to *F. japonica* (Gammon et al., 2007).

found in our populations. These were likely founded by single propagules, and all were unique genets not represented in neighboring areas, thus suggesting sexually produced seed and not rhizomes as the most common dispersal propagule.

If patch expansion were driven by sexual reproduction, then adjacent ramets in a patch would often be different genets. Alternatively, if clonal growth drives expansion, adjacent ramets would often be samples of the same genet. Examining samples every 2 to 5 m in all contiguous stands along our transects, we found that 24 of 42 adjacent pairs had identical genotypes, indicating extensive clonal growth within stands.

F. sachalinensis-specific marker—In addition to our three populations, other samples from Massachusetts, including *F. sachalinensis* and *F. japonica*, were used to further test locus KW6. The primers for the KW6 marker amplified a dominant fragment in all of our local specimens of *F. sachalinensis*. The fragment was rare in all collections that appeared to be *F. japonica* (Table 4). The reverse primer was suspected to be specific to *F. sachalinensis* because a new primer placed closer to the forward primer amplified this fragment in all samples. We tested the supposition that the fragment was specific to *F. sachalinensis* by screening samples from Britain and from Washington in the western U.S. that have been identified as pure *F. japonica*, *F. sachalinensis*, or F1 hybrid *F.* ×*bohemica* (Table 4). *Fallopia japonica* produced no visible amplification product, while plants identified as *F. sachalinensis* or hybrids from these regions produced the fragment. Because this fragment was consistently amplified from all British and Washington samples that have the *F. sachalinensis* genome as well as all *F. sachalinensis* from our region, we used this marker as an indicator of potential hybridization in our local populations.

The amplification product for KW6 was detected in 15 of the 26 different genets (Table 3; shown in bold). Interestingly, 14 of these 15 genets were male, suggesting that most males are hybrids. Conversely, eight of 11 genotypes that lacked the fragment were female, and these sex-specific differences were significant ($P = 0.0021$). These data suggest that although the populations are composed mostly of hybrid males and *F. j.* var. *japonica* females, they also contain males and females of both *F. j.* var. *japonica* and *F.* ×*bohemica*.

DISCUSSION

Fallopia japonica is notorious for its capacity to spread vegetatively by extensive rhizomes (Bailey, 1994; Beerling et al., 1994). Its astounding ability to spread asexually was substantiated in Britain, where 150 British *F. japonica* samples produced an identical RAPD profile, suggesting the presence of a single widespread clone (Hollingsworth and Bailey, 2000). Samples from the U.S. and three other European countries were also included among those samples and showed the same RAPD profile, suggesting that they too are part of this massive clone. However, only a few samples from the U.S. were included in that study, leaving questions about the genetic diversity of Japanese knotweed in the U.S.

Until recently, evidence of sexual reproduction of invasive *F. japonica* has been lacking because in Britain, genetic diversity is low (Hollingsworth and Bailey, 2000) and seed production appears restricted to hybridizations with related species (Bailey, 1994), and in the U.S., achenes on female plants were often empty and seedlings rarely or never survived to maturity (Locandro, 1973; Seiger, 1997). Consequently, rhizomes have been considered the primary and perhaps the sole means of reproduction and dispersal in introduced ranges (Shaw and Seiger, 2002; Zika and Jacobson, 2003), and discussions of management have largely neglected the impact of seed dispersal and genetic diversity (Soll, 2004; Talmage and Kiviat, 2004; McHugh, 2006). While populations of wild *F. japonica* sensu lato in the northeast U.S. have now been shown to produce large quantities of seed with high germination rates (Forman and Kesseli, 2003; Bram and McNair, 2004), there have been no genetic studies, until now, demonstrating the consequences of this sexual reproduction, the extent of genetic diversity within populations, or the contribution of sexual reproduction to the spread of this species within the U.S.

Soll (2004, p. 5) noted, “Should extensive sexual reproduction be confirmed in the field, it would certainly alter the strategy for landscape level control projects.” With this new evidence of sexual reproduction and genetic diversity, management for *F. japonica* should no longer ignore seed as a dispersal propagule. In addition, biological control methods designed for clonal species may be compromised if populations with large amounts of variation are able to adapt (Lee, 2002), or they may fail altogether if studies testing the efficacy of controlling agents do not screen appropriately diverse host accessions. Indeed, contrary to results obtained with accessions from Europe, preliminary results have found that U.S. populations of knotweed yield varying responses to infections from pathogens (Richard Shaw, CABI UK Centre, personal communication). Taken together, these differences in population structure and life histories suggest that management

strategies different from those being developed in Europe may be needed in the U.S.

Our data demonstrate that unlike the situation in Europe, both seed dispersal and vegetative growth contribute to the spread of this species in the northeast U.S., a pattern similar to that described in Japanese populations (Zhou et al., 2003). While there are several possible mechanisms that could create the structure seen in our study sites, patches were most likely established by sexually produced, dispersed seeds followed by expansion through vegetative growth. Given the high capacity for vegetative spread, it is likely that some genets were introduced via rhizome fragments, but several observations point toward sexual reproduction and seed dispersal. First, seeds are abundant and germination is high (Forman and Kesseli, 2003; Bram and McNair, 2004). Second, seedlings have been observed in the wild in Massachusetts (Forman and Kesseli, 2003). Third, there are several genets at each site. Fourth, physically separated patches were most often composed of unique genets found in no other patch within a population. Finally, all genets in the populations could be derived from a small number of founders by recombination. Of course, there was also significant evidence of vegetative growth within populations because more than half of all pairs of adjacent ramets sampled within contiguous stands were genetically identical. This combination of seed dispersal and clonal growth has also been inferred by genetic evidence in other invasive plant species including Canada goldenrod, *Solidago canadensis* (Dong et al., 2006) and kudzu, *Pueraria lobata* (Pappert et al., 2000).

Significantly, the one genet (genotype B) found repeatedly in all three Massachusetts populations matched the genetic profile of the aggressive female clone of *F. japonica* from Britain (Table 3). While the distribution of this genet in other regions of the U.S. is not known, the existence of this clone in our populations is noteworthy and has two likely explanations. Widespread distribution in naturalized populations may simply indicate that this clone had a widespread distribution as an ornamental and subsequently escaped. Alternatively, this clone may be more successful in reproducing asexually. Interestingly, Barney (2006) identified 151 herbaria collections indicating that Japanese knotweed was commonly found as an escaped ornamental, which may support the first hypothesis. Obviously, taxa that are largely clonal or have limited diversity can be successful invaders. Knotweeds in Europe are prime examples, but others such as the common reed (*Phragmites australis*) with its extensive clonal growth and single chloroplast haplotype have successfully invaded much of North America (Saltonstall, 2002, 2003). Further investigation is required to assess the occurrence and impact of *F. japonica* genotype B in other regions of North America.

Our U.S. populations display a diversity and structure similar to that described in Asia, yet our populations appear to involve the extensive hybridization prevalent in Europe. Hybridization events, however, do appear to occur in Asia. Inamura et al. (2000) found that accessions from Japan, defined by morphological characters as different species and varieties of *Fallopia*, did not form monophyletic groups based on chloroplast sequences. Indeed, one population of *F. sachalinensis* grouped with accessions of *F. japonica* var. *uzensis*. In Korea, Kim and Park (2000) identified one population that appeared to have hybrids involving three species including *F. japonica* and *F. sachalinensis*. Without additional data, it is difficult to assess the relative contributions of hybridization among species and sexual reproduction within diverse accessions of *F. j.* var. *japonica* to the genetic diversity observed in our populations. Because 14 of 17 male genotypes amplified the *F. sachalinensis*-specific fragment (KW6), most male plants appear to be hybrid, supporting findings in Europe (Bailey, 1994). Contrary to British studies, however, both hybrid and nonhybrid males were detected as well as multiple male and female genotypes of *F. j.* var. *japonica*. Because all of the females in our populations produced abundant seeds, these data also indicate that it is not just hybrids that are fertile as has been surmised (Soll, 2004). Together, these data point to a more complicated population structure that likely involves sexual reproduction within *F. j.* var. *japonica*, hybridization between *F. japonica* and *F. sachalinensis*, and introgression. Using both morphological characters and genetic markers, Gammon et al. (2007) found similar evidence of this complicated reproductive system in New England populations. The *sachalinensis*-specific marker used in this study, along with other taxon-specific markers, may be valuable in determining the extent of hybridization within other regions in the U.S., especially because identifying species by morphological traits has proven difficult (Gammon et al., 2007).

In the present study, we have shown for the first time that populations of knotweed in the U.S. are genetically diverse and not purely clonal as previously assumed. The population structure found in our region is compatible with the idea of sexual reproduction and seed dispersal along with hybridization and introgression as major processes in the life history of these taxa in the U.S. These complex reproductive dynamics, the polyploid nature of *Fallopia* spp., and their widespread geographic distribution lead us to believe that the invasive potential of these species is far reaching and, unfortunately, has not yet been realized. This new information about the reproductive dynamics indicates that the invasion biology of knotweeds in the U.S. differs from that in Europe and demonstrates the need for regional studies before broad generalizations can be developed for dynamic, likely rapidly evolving taxa. We currently know nothing about the genetic composition of Japanese knotweed populations in other regions of the U.S., and given the drastic differences between Britain and the eastern U.S., it would be imprudent to generalize about all U.S. populations. Indeed, the differences observed should remind researchers that invasive taxa, particularly those invading different regions of the world, may have distinct life history patterns and may not follow similar trajectories; that molecular analyses may be needed to dissect these divergent ecological patterns; and that management strategies may need to be individually tailored to the local or regional dynamics.

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