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Polygonum cuspidatum (Polygonaceae) Genetic Diversity in a Small Region of Eastern Kentucky

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ABSTRACT

Polygonum cuspidatum Sieb. & Zucc. is an invasive species that has spread across Britain, Europe, Canada, and the United States. Prior to this research, no analysis of genetic diversity within, or among, populations had been conducted in the United States, although population genetic diversity had been examined in Britain. An analysis of genetic diversity was carried out using Polymerase Chain Reaction (PCR)-based DNA fingerprinting on plant populations along two creeks in Rowan County, Kentucky. One creek was sampled at one-meter intervals and the other at intervals of one to nine km. The results of this analysis indicate that the plants along both of these creeks are genetically dissimilar and not clonal. The data did not support the hypothesis that *P. cuspidatum* is reproducing and dispersing by asexual means.

KEY WORDS: DNA fingerprinting; *Fallopia japonica*; genetic diversity; Japanese knotweed; *Polygonum cuspidatum*; RAPD analysis

INTRODUCTION

Japanese knotweed (*Polygonum cuspidatum* Sieb. & Zucc., synonyms *Fallopia japonica* (Houtt.) R. Decr., *Reynoutria japonica* Houtt.) is an invasive plant species introduced into northeastern North America in the late 1800s. Since that time, it has spread across the United States and into Canada. It is widely distributed in the northeastern United States, and its distribution decreases as one moves south and west of Kentucky (USDA, NRCS 2006). In the United States, *P. cuspidatum* is listed as a noxious weed in 35 states, including Kentucky. *P. cuspidatum* prefers a riparian habitat along streams and ditches but can grow almost anywhere (USDA, NRCS 2006). Because *P. cuspidatum* has a rapid growth rate and produces an extensive rhizome system, it rapidly outcompetes native species, forming dense stands along stream banks thus making it a threat to native plant populations. Furthermore, it is an economic burden to those entities that maintain roadsides, ditches, and streams.

A plant's mode of reproduction significantly impacts that plant's mechanism for dispersal. In the case of an invasive plant such as *P. cuspidatum,* understanding the mechanism of dispersal can lead to more informed and effective management practices. *P. cuspidatum* exhibits both asexual and sexual modes of reproduction. *P. cuspidatum* has a tremendous capacity for vegetative propagation from rhizomes and aerial stem fragments. The rhizome has been observed to grow horizontally for 15–20 m (Locandro 1973; Conolly 1977), and a fragment of rhizome as little as 0.7 g in size can produce a new plant (Brock and Wade 1992). *P. cuspidatum* also can regenerate from aerial stem tissue. DeWaal (2001) found that node-containing aerial stem sections 40 mm-long produced new shoots and adventitious roots when placed on moist soil.

In terms of sexual reproduction, *P. cuspidatum* is dioecious with the fruits being achenes. It also has been described by Beerling et al. (1994) as being gynodioecious. Bailey (1994) reported that the *P. cuspidatum* populations in Britain contained only male-sterile and hermaphroditic plants. No pure-bred seeds have been found in Britain (Beerling et al. 1994). *P. cuspidatum* can hybridize with the closely related species *F. sachalinensis* and *F. baldschuanica* (Beerling et al. 1994). The implication of these studies is that reproduction and dispersal are the results of vegetative propagation, at least in Britain.

In contrast, *P. cuspidatum* produces large quantities of viable seeds in its native range in Japan (Maruta 1983). Survival of first-year

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seedlings is heavily dependent on their ability to attain a "critical dry matter production" due to environmental stresses. Furthermore, patch establishment in Japan was found to be the result of seed dispersal as opposed to vegetative propagation, while patch development/expansion was found to be a combination of these two modes of reproduction (Zhou et al. 2003).

In the United States, large quantities of seeds can be produced by *P. cuspidatum,* but there are conflicting reports regarding the viability of these seeds and survivability of the seedlings. Locandro (1973) reported that in the presence of fertile males, seeds were produced that germinated, but that these seedlings did not survive the first year. In the absence of fertile males, seeds were produced, but the achenes were empty. Seiger (1993) reported that seed germination rates were low (from 10–63%, depending on their period of dormancy). Seedling survival was low in the laboratory, and none was observed in the wild. It was the conclusion of Locandro (1973) and Seiger (1993) that sexual reproduction played a very minor role in the reproduction and dispersal of *P. cuspidatum* in the United States.

In contrast, Forman and Kesseli (2003) reported high mean germination rates of 65–79% for *P. cuspidatum.* These germination rates were dependent upon treatment with some seed sets showing 100% viability. Survival of these seedlings ranged from 0–100% with a mean of 65%. Similarly, Bram and McNair (2004) reported a high degree of seed viability as measured by seed germination. They found that seed germinability was dependent upon maturation of the seeds. Less than 10% of seeds produced early in the season germinated, while germination was greater than 90% for seeds formed later in the season. Forman and Kesseli (2003) and Bram and McNair (2004) concluded that sexual reproduction may play a very important part in the reproduction and dispersal of this species.

Observations of the lack of male-fertile plants in Britain imply that vegetative propagation is the primary means for reproduction and dispersal, at least in some locations. If this hypothesis is correct, then all plants within a population should be genetically identical to each other. In Britain, this hypothesis has been tested and confirmed using molecular techniques. DNA extracted from plants across Britain was subjected to Random Amplified Polymorphic DNA (RAPD) analysis. Hollingsworth et al. (1998) and Hollingsworth and Bailey (2000) showed that the vast majority of *P. cuspidatum* populations in Britain were genetically identical and thus clonal. The populations that were not genetically identical to the rest were found to be hybrids between *P. cuspidatum* and *F. sachalinensis* (Hollingsworth et al. 1998). A study from the Czech Republic by Bímová et al. (2004) came to a similar conclusion based on isozyme analysis; vegetative propagation is the primary means of reproduction, but sexual reproduction can occur.

Considering the contradictory reports of seed germinability and seedling survival for *P. cuspidatum* plants in the United States, the role for asexual compared with sexual reproduction is not clear. Furthermore, no genetic analysis of *P. cuspidatum* populations in the United States has been performed.

In order to determine the degree of genetic diversity of *P. cuspidatum* populations in the United States, *P. cuspidatum* populations were sampled in a localized region of eastern Kentucky. RAPD analysis was utilized to genetically characterize plants along two streams.

MATERIALS AND METHODS

Plant Material

Plant samples for this study were collected along two streams, Evans Branch and Triplett Creek, which flow through Morehead, Kentucky. Evans Branch is a tributary of Triplett Creek (Figure 1). Sampling for Evans Branch was carried out where the stream runs through a parking area before emptying into Triplett Creek (Figure 1). This patch of *P. cuspidatum* formed one continuous stand, approximately 20 m long. Two samples were collected at 1-m intervals along a linear transect parallel to the creek. Sampling for Triplett Creek was carried out at intervals of 1 to 9 km and included areas inside and outside of the Morehead city limits but within Rowan County (Figure 1). The patches of *P. cuspidatum* along Triplett Creek were discontinuous. Two leaf samples were collected at each site, but only one was used for the genetic analysis.

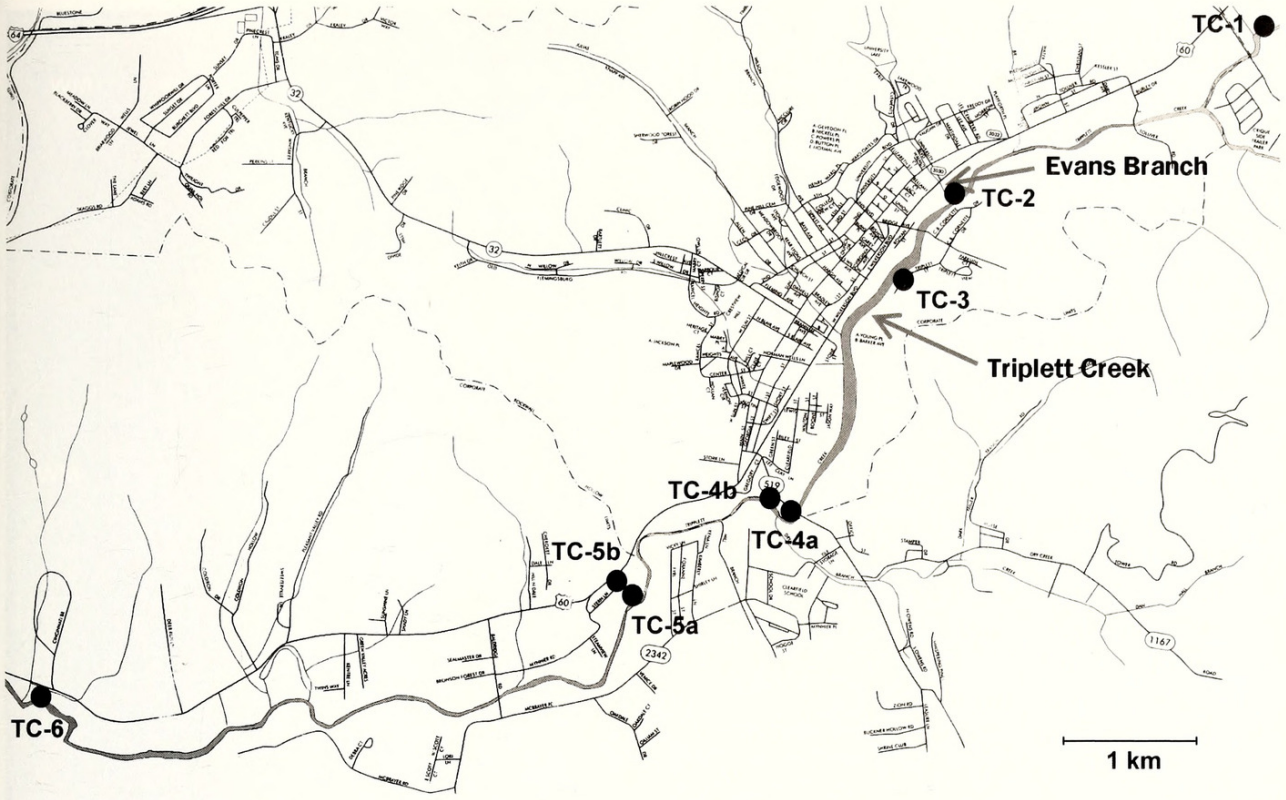

Figure 1. Map of Evans Branch with respect to Triplett Creek and the sampling sites along Triplett Creek. Samples were collected both upstream and downstream of Morehead, Kentucky, USA.

DNA Extraction

DNA extraction was carried out using the Plant DNAzol reagent (Invitrogen, Carlsbad, California, USA). When samples were processed immediately, DNA extraction was carried out according to instructions by the manufacturer. Some plant samples were not processed until the plant material had become dry. In this case, the manufacturer's instructions were altered so that a higher volume of Plant DNAzol reagent (3- or 4-fold more) was used per gram of tissue to compensate for water loss. DNA was quantified by spectrophotometric absorbance at 260 nm.

RAPD Analysis

RAPD amplification reactions were performed in a total volume of 20 μL with the final concentrations of 1.25 units Thermo-Start DNA polymerase, 3.0 mM $MgCl_2$, 0.2 mM dNTPs, 10 ng plant DNA, and 3 or 30 μM primer. PCR reagents were supplied in Thermo-Start PCR Master Mix (ABgene, Surrey, United Kingdom) supplemented with additional $MgCl_2$.

Table 1. Sequences of the RAPD primers used for PCR.

Primer	Sequence 5′-3′
OPA-06	GGT CCC TGA C
OPA-16	AGC CAG CGA A
OPF-16	GGA GTA CTG G
RAPD-A01	CAG GCC CTT C
RAPD-A09	GGG TAA CGC C

More than thirty 10-mer oligonucleotide primers were tested including those from the Operon A and F sets. The following primers were chosen for their ability to produce an appropriate number of reproducible bands: OPA-6, OPA-16, OPF-16, RAPD-A01, and RAPD-A09. Sequences of the primers are given in Table 1.

Amplification was carried out essentially as reported by Congiu et al. (2000) using an Applied Biosystems (Foster City, California, USA) GeneAmp 7200 thermal cycler set to the following parameters: (1) 3 min of enzyme activation at 94°C; (2) 45 cycles of: 40 s at 94°C, 70 s at 48°C, 120 s at 72°C; (3) 7 min at 72°C. Each reaction was carried out in triplicate.

PCR products and molecular weight standards were separated on 1.6% agarose gels and stained with ethidium bromide. Images of

the gels were captured using the Kodak (Rochester, New York, USA) EDAS 290 1D system and were analyzed using Phoretix 1 D software (Nonlinear Dynamics, Newcastle-Upon Tyne, UK). Bands were scored only if they were present in two of the three replicates. The resulting RAPD markers for each plant were pooled and used to generate dendograms with Phoretix 1 D software by the unweighted pair group method with arithmetic averaging (UPGMA).

Controls

Several plant samples were collected for use as outgroups. The LR sample was taken from the Lower Lick Fork, a tributary of the Licking River into which Triplett Creek flows. The LR sample was taken approximately 40 km from the Triplett Creek samples. The NW sample was taken from a site along North Wilson Street in Morehead that was geographically isolated from both Evans Branch and Triplett Creek.

To gauge the amount of genetic similarity that could be detected, two leaves from the same plant (P1L1 and P1L2) were collected and analyzed as if they were independent plants. The plants were collected from a site geographically separated from both Evans Branch and Triplett Creek. Leaves from two separate plants were analyzed in this way, and leaves from the same plant were always grouped together in preliminary analyses. Only one plant was included in the dendrograms presented.

RESULTS

Evans Branch

Two samples from Evans Branch failed to yield useable DNA (sample #5 and #6) and were not included in the analysis. Sample #4 was not adequately amplified by two primers, so it also was excluded from analysis. RAPD analysis along Evans Branch using five primers yielded 61 RAPD markers, of which 95% were polymorphic. Markers ranged in size from 120 bp to 1500 bp. A sample gel is shown in Figure 2. The number of RAPD markers across all primers ranged from 10–15.

To determine the degree of genetic similarity that might be expected from clonal plants, two leaf samples from the same plant were

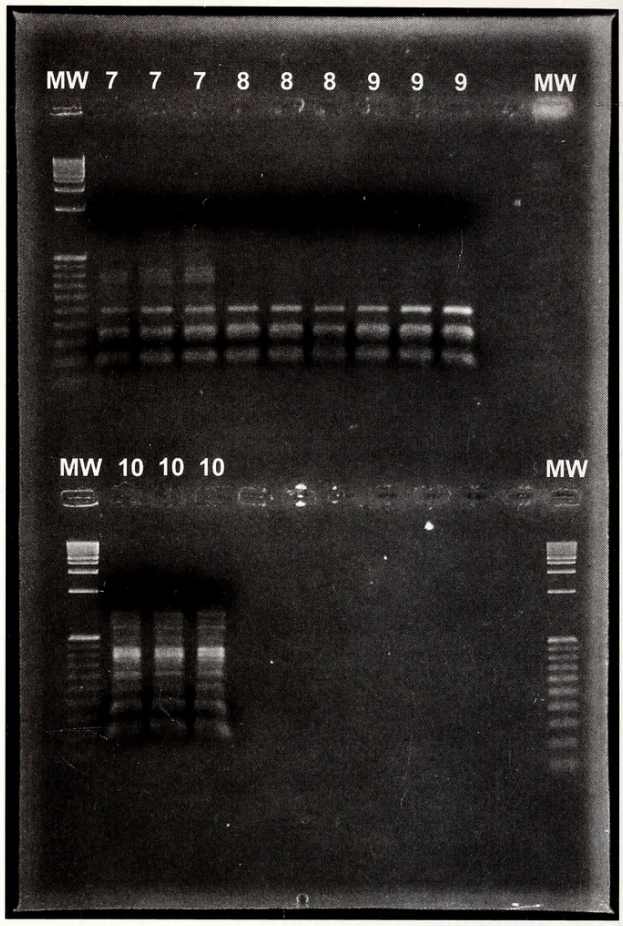

Figure 2. Electrophoretic gel illustrating the genetic diversity of the plant samples.

included in the analysis as if they were taken from independent plants. These plants were collected from a location that was geographically separated from Evans Branch and Triplett Creek. The dendogram produced for plants along Evans Branch (Figure 3A) revealed two genetically distinct groups; one at each end of the sampling area. As expected, the control samples (that were known to be genetically identical) grouped with each other and were separate from the rest of the plants along Evans Branch (Figure 3A). Similar clustering was observed when the data were analyzed using a neighbor-joining algorithm (data not shown). Samples #2 and #3 were the only samples that were genetically indistinguishable from each other (and thus presumed to be clonal). All of the other plants sampled along Evans Branch (at 1-m intervals) were more diverse from each other than the known clonal samples, indicating that they were not genetically identical. Relative plant positions

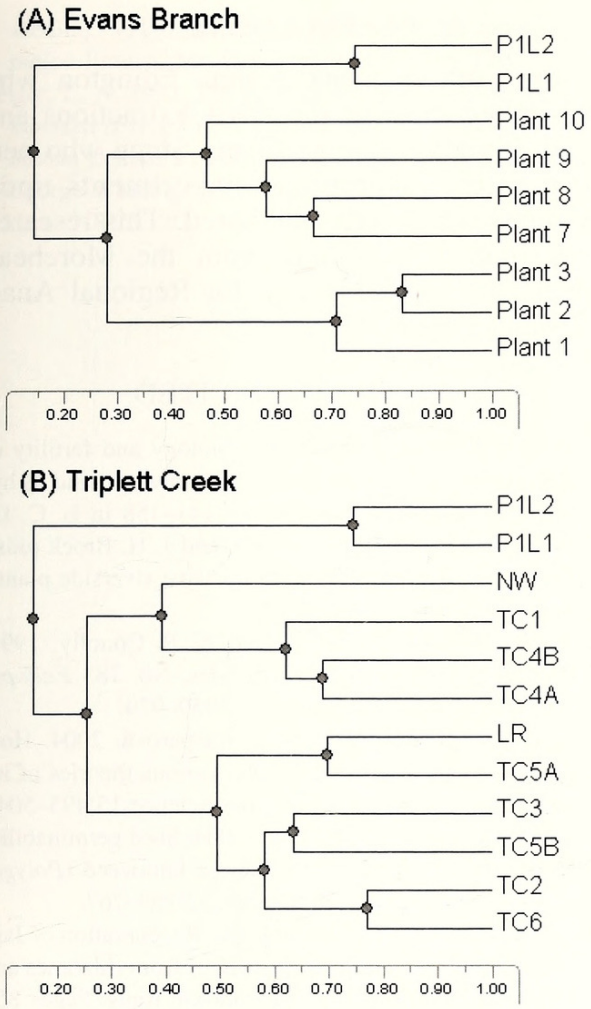

Figure 3. UPGMA dendogram, based on RAPD data, for individuals of *P. cuspidatum* collected (A) along Evans Branch and (B) along Triplett Creek.

at the study site appeared to influence clustering.

Triplett Creek

Samples were collected at intervals along Triplett Creek and at two sites distant from the creek. One site (NW) was from a ditch that was about 0.8 km away from the creek. The other was from the Lower Lick Fork, a tributary of the Licking River (LR) into which Triplett Creek flows but about 40 km away. RAPD analysis of these samples using five primers yielded 80 markers, 97% of which were polymorphic. Markers ranged in size from 70 bp to 2300 bp. The number of RAPD markers across all primers ranged from 13–21. The dendogram produced for the plants along Triplett Creek indicated that only two plants were clonal (TC2 and TC6; Figure 3B). Sample TC2 was approximately 9 km upstream of TC6. None of the plants from neighboring sites grouped together with the exception of TC4a and TC4b. One supposed outgroup, LR, grouped more closely with Triplett Creek samples than did Triplett Creek samples group with each other. NW also grouped with the Triplett Creek samples, although not as closely. The control samples (known to be clonal) grouped with each other and were separate from the rest of the plants along Triplett Creek (Figure 3B), as expected. Similar clustering was observed when the data were analyzed using a neighbor-joining algorithm (data not shown). Relative plant positions did not appear to influence clustering.

DISCUSSION

Although *P. cuspidatum* observed and sampled along Evans Branch form a single, large population, only two genetically identical samples were observed (located 1 m apart). The same lack of genetic similarity was observed for the plant populations along Triplett Creek. It is apparent that multiple genotypes of *P. cuspidatum* exist in the United States and even along approximately 12 km of Triplett Creek.

These results stand in sharp contrast with the results from populations sampled throughout Britain (Hollingsworth and Bailey 2000) where all samples were found to be genetically identical. Hollingsworth and Bailey (2000) attributed this clonal relationship to the observation that plants in Britain are male-sterile (Beerling et al., 1994). Forman and Kesseli (2003) and Bram and McNair (2004) provide evidence that *P. cuspidatum* plants in the United States are fertile producing viable seeds that can over-winter. In Japan, Zhou et al. (2003) have shown that seed dispersal is a mechanism by which *P. cuspidatum* can spread.

In the United States, *P. cuspidatum* is found primarily in locations that have been disturbed by humans; either humans have purposefully planted *P. cuspidatum* or humans have accidentally transferred the plant in the process of transferring soil from one place to another. The original source(s) of *P. cuspidatum* in Rowan County is unknown. Along the stretch of Evan's Branch that was sampled, soil was brought in from at least one remote site when a parking lot was built. The origin of the

plants along Triplett Creek is not known; although all of the areas sampled had been disturbed by humans. The plant populations along Triplett Creek could have formed from rhizome fragments or seeds in transported soil, seeds carried by air or water, or stem fragments carried by water. If the dispersal giving rise to these populations were by fragmentation or extension of the rhizome system (thus, asexual), then it would be expected that plants positioned next to each other would be genetically identical (clonal). Only two of the plants along Triplett Creek were found to be clonal, and there was no clustering of plants based on their relative location. This excludes reproduction and dispersal by an asexual mechanism. The data are consistent with the hypothesis that diverse genotypes are the result of diverse genetic backgrounds as the result of introduction from multiple sources, suggesting genetic diversity extends beyond Rowan County, Kentucky. It is also possible that these plants are reproducing sexually, but given the great distance between the two clonal samples along Triplett Creek, the evidence does not support this hypothesis.

The data are different along Evans Branch. Plants 2 and 3 were found to be clonal. There was a clustering of the genotypes based on the plants' relative locations; plants on opposite ends of the transect grouped separately. The two clonal samples were only 1 m apart and may represent asexual reproduction by either fragmentation or rhizome extension. The genotypic clustering within the population may indicate that neighboring plants are exchanging pollen and are in-breeding. Thus, along Evan's Branch, there is some evidence for sexual reproduction as a dispersal mechanism.

The populations of *P. cuspidatum* studied were found to be genetically diverse, even within one county of Kentucky. Thus, multiple genotypes exist in the United States. It is interesting to speculate that this genetic diversity might be beneficial for population expansion given the wide distribution of *P. cuspidatum* in the United States (USDA, NRCS 2006). Little evidence of dispersal by asexual reproduction was observed along either creek studied; however, there was some evidence for sexual reproduction among neighboring plants within a large stand of knotweed plants along Evans Branch.

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