Genetic diversity of *Pueraria lobata* (kudzu) and closely related taxa as revealed by inter-simple sequence repeat analysis

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Received 16 June 2004
Revised version accepted 24 February 2005

Summary

Kudzu (*Pueraria lobata*) is a noxious weed infesting some areas of the USA. Knowledge of its genetic variation in both native and invasive areas can lead to effective biological control measures. Inter-simple sequence repeat (ISSR) variations were studied in *P. lobata* and its four closely related congeneric species (*P. edulis*, *P. montana*, *P. phaseoloides* and *P. thomsoni*). ISSR results allowed a clear separation of these five species. For *P. lobata*, 108 plants from China and USA were analysed. The samples from the US were genetically closer to Chinese *P. lobata* populations than to other congeneric populations. High genetic differentiation was found for *P. lobata*, *P. montana* and *P. thomsoni* in Chinese samples. High genetic diversity and low population differentiation was found in *P. lobata* samples of the US. This supports the hypothesis of multiple introductions into the USA from different sources in Japan or China, followed by subsequent gene exchange and recombination.

Keywords: genetic variation, invasive weed, ISSR, kudzu, *Pueraria*.

SUN JH, LI Z-C, JEWETT DK, BRITTON KO, YE WH & GE X-J (2005) Genetic diversity of *Pueraria lobata* (kudzu) and closely related taxa as revealed by inter-simple sequence repeat analysis. *Weed Research* **45**, 255–260.

Introduction

Pueraria lobata (Willd.) Ohwi [kudzu, Leguminosae, synonymous *P. montana* var. *lobata* (Willd.) Maesen and Almeida] is a perennial, semi-woody, climbing legume in the tribe Phaseoleae Benth., subtribe Glycininae Benth. (van der Maesen, 1985). The large purple flowers of *Pueraria* are produced in relative abundance with a sweet aroma. Its corolla is papilionaceous and 14–20 mm long. The mating system of *P. lobata* includes both sexual reproduction through bee pollination and asexual reproduction through rhizome spread. *P. lobata* is native to China, Korea and Japan; it was introduced into the USA as an ornamental plant in 1876 from Japan, at the Philadelphia Centennial Exposition. During the first half of the 20th century, *c.* 134 760 ha were planted throughout the south-eastern US as forage and for erosion control (Britton *et al.*, 2002). *P. lobata* replaces existing vegetation resulting in ecological and economic losses. Where productive forest land is infested, lost productivity has been estimated at \$118 ha^{-1} $year^{-1}$ (Britton *et al.*, 2002). In 1998, *P. lobata* was listed by the United States Congress as a Federal Noxious Weed. No effective and efficient control measures have been found so far and herbicides are still the primary management tool. The most effective herbicides are not selective and cannot be used near waterways. Moreover, unless a landowner owns the entire *P. lobata* 'patch', control is often only temporary, because of subsequent reinvasion. These difficulties led to an investigation into the potential for biological control of *P. lobata*, using insects and diseases from China. Pemberton (1988) reported abundant natural enemies of *P. lobata* in China and Korea.

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Approximately 17 species in the genus *Pueraria* are recognized globally (van der Maesen, 1985). However, the taxonomical treatment is confused; different scientific names are often used in the literature to refer to kudzu in the US, such as *Pueraria lobata* (Willd.) Ohwi, *Pueraria montana* (Lour.) Merr., *Pueraria thomsoni* (Benth) Maesen, or *Pueraria montana* var. *lobata* (Willd.) Maesen and Almeida (Ward, 1998). In China, these taxa are sympatric throughout part of their ranges and are difficult to distinguish morphologically (Wu *et al.*, 1994). Morphological characteristics that had been used to differentiate *P. lobata* from *P. montana* and *P. thomsoni* are lobed leaflets and the size of wing and keel petals, all of which can be quite variable. Furthermore, there are possible hybrids between these related *Pueraria* taxa (van der Maesen, 1985). *P. lobata* is considered the most common species and occurs throughout China, except in Xinjiang, Qinghai and Tibet. Moreover, *P. lobata* is also found in Japan. van der Maesen (1985) considered these species as varieties.

Incomplete systematic resolution may be an obstacle for developing an effective biological control programme for this weed, as confusion with its related taxa occurs in China. The identity of populations in the US and Asia has not been reconciled, which may present problems in matching potential biological control agents with their target (Pappert *et al.*, 2000; Jewett *et al.*, 2003). Although van der Maesen (1985) concluded that only one variety (*P. montana* var. *lobata*) was widely distributed outside Asia, he used herbarium materials vouchered before 1985 to come to that conclusion. Studies by Jewett *et al.* (2003) using random-amplified polymorphic DNA (RAPD) supported the conclusion of Pappert *et al.* (2000) that the population of *P. lobata* in the US demonstrates considerable genetic variation. However, this study was inconclusive regarding the genetic relationship between *P. lobata* in the US and *P. lobata*, *P. montana* and *P. thomsoni* in China, because of limited sampling.

Pueraria lobata appears to be appropriate for classical biological control, as this naturalized weed lacks natural enemies capable of controlling it in the US (Britton *et al.*, 2002). However, in order to successfully implement a classical biological control programme, it is necessary to gain as much knowledge as possible about the genetic diversity of both introduced and native populations and the genetic relationship among related taxa. Molecular genetic tools have been successfully applied to the study of many invasive species. In the past decade, inter-simple sequence repeat (ISSR) polymorphism markers were developed to study the genetic diversity of natural populations, including several invasive plant species (Meekins *et al.*, 2001; Mengistu & Messersmith, 2002; Ash *et al.*, 2003). These have been shown to provide adequate information for resolving phylogenetic relationships among closely related species (Mort *et al.*, 2003; Pharmawati *et al.*, 2004).

In this study, ISSR analysis was used to investigate the genetic variation of *P. lobata* and closely related species *P. montana*, *P. thomsoni*, *P. edulis* and *P. phaseoloides* with the aims of: (i) determining its utility to discriminate closely related *Pueraria* species, and (ii) determining the genetic diversity of *P. lobata* in both the US and China, in order to facilitate the identification of effective biological control agents for this weed in the US.

Materials and methods

Plant materials and DNA extraction

A total of 260 plant samples were collected both from China (Fujian, Guangdong, Jiangxi, Liaoning, Yunnan and Zhejiang Province) and from 19 states of the USA (AL, AR, DE, FL, GA, IN, KY, MD, MO, MS, NC, OH, OK, PA, SC, TN, TX, VA, WV) (Table 1). In China, *P. lobata* was represented by two populations, *P. thomsoni* by three populations, *P. montana* by five populations, and *P. edulis* and *P. phaseoloides* by a single population each. Because asexual reproduction occurs by rhizome, individuals that were at least 10 m apart from each other were sampled, to reduce the probability of sampling the same clone. Young leaves were collected and dried in silica gel until further processing. Genomic DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method of Doyle (1991) and purified using a DNA clean kit (Dingguo Biotech, Beijing, China).

PCR amplification

Nuclear DNA was polymerase chain reaction (PCR)-amplified using ISSR primers from the University of British Columbia primer set 9. Following an initial screening of 100 primers, 11 (UBC no. 807, 808, 809, 811, 834, 835, 836, 840, 842, 881 and 888) that yielded maximum numbers of reliable and reproducible polymorphisms were selected to analyse the populations. PCR was carried out in a total volume of 20 μL consisting of 20 ng of template DNA, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 2% formamide, 0.2 μM primer, 1.5 U of *Taq* polymerase and double-distilled water on a MJ Research 96-well thermal cycler with hot bonnet (Bio-rad, Waltham, MA, USA), following the protocol of Ge and Sun (1999). PCR products were separated by gel electrophoresis on 2.0% agarose gels in 0.5x TBE buffer and visualized using ethidium bromide staining (0.1 μg mL^{-1}). DNA fragments were visualized by image analysis software for gel documentation (LabWorks Software, Version 3.0; UVP, Upland, CA, USA).

Table 1 Sample location and number of samples of each species of *Pueraria* studied

Species	Population identifier	Sample number	Location	Longitude	Latitude (N)
P. edulis	P.edulis	22	Lijiang, Yunnan	100°E	27°
P. lobata	PlCN1	18	Anshan, Liaoning	123°E	41°
	PlCN2	9	Leqing, Zhejiang	120°E	28°
P. thomsoni	Pt1	16	Guangzhou, Guangdong	113°E	23°
	Pt2	10	Lijiang, Yunnan	100°E	27°
	Pt3	6	Dongxiang, Jiangxi	116°E	28°
P. montana	Pm1	23	Guangzhou, Guangdong	113°E	23°
	Pm2	16	Boluo, Guangdong	114°E	23°
	Pm3	12	Sanming, Fujian	117°E	26°
	Pm4	14	Luoyuan, Fujian	119°E	26°
	Pm5	10	Yongan, Fujian	117°E	26°
P. phaseoloides	Phaseoloides	23	Guangzhou, Guangdong	113°E	23°
USA samples	US1	4	Colbert, AL	87°W	34°
	US2	2	Crawford, AR	94°W	35°
	US3	3	Kent, DE	75°W	39°
	US4	4	Alachua, FL	82°W	29°
	US5	11	Burke, Fannin and Macon, GA	81°–84°W	32°–34°
	US6	2	Warrick, IN	87°W	37°–38°
	US7	9	Bell and Bracken, KY	83°–84°W	36°–38°
	US8	1	Anne Arundel, MD	76°W	38°
	US9	6	Butler, Dunklin, Ray, MO	90°–93°W	35°–39°
	US10	2	Tippah, MS	88°W	34°
	US11	10	Davie, New Hanover, NC	77°–80°W	34°–36°
	US12	1	Muskingham, OH	82°W	39°
	US13	1	Okfuskee, OK	96°W	35°
	US14	2	Lebanon, PA	76°W	40°
	US15	5	Berkely, Calhoun, SC	80°W	33°
	US16	5	Haywood, Weekley, TN	88°–89°W	35°–36°
	US17	2	Smith, TX	95°W	32°
	US18	5	Madison, Westmoreland, VA	76°–78°W	38°
	US19	6	Putnam, Roane, WV	81°–82°W	38°

Data analysis

Only bands that could be unambiguously scored across all the population samples were used in this study. ISSR profiles were scored for each individual as discrete characters (presence or absence of the amplified products). Interpopulational relationships were examined by Nei's genetic distance (D) between pairs of populations (Nei, 1972). An unweighted pair-group method using arithmetic average (UPGMA) dendrogram was constructed based on the matrix of genetic distance using the SAHN-clustering and TREE programs from NTSYS-pc 2.0 (Rohlf, 1998). Components of variance partitioned within and between populations were estimated using analysis of molecular variance (AMOVA). AMOVA analyses were performed using the ARLEQUIN 2.000 program (Schneider *et al.*, 2000). The number of permutations for significant testing was set at 1000.

Shannon's diversity index, which is less influenced by sample size, was used to compare levels of genetic diversity in Chinese and the US *P. lobata*, by calculating $H_o = -\sum p_i \log_2 p_i$ (Lewontin, 1972), in which p_i is the frequency of a given ISSR fragment.

Results and discussion

A total of 182 different ISSR bands were scored, corresponding to an average of 16 bands per primer. Of the 143 bands that occurred in the US material, 104 were shared with the Chinese *P. lobata* material. AMOVAS showed high genetic differentiation (significant Φ_{ST} values) for all the group comparisons (Table 2).

The species delimitation

AMOVA showed a remarkable diversity among the five species (35.6%; Table 2). The cluster analysis (UPGMA) indicated that all the species were well separated (Fig. 1). Although it is difficult to identify species of *Pueraria* morphologically, all populations of each species appeared clustered together. The ISSR analysis matched exactly the taxonomic diagnostic criteria. The two Chinese *P. lobata* populations were grouped with the 19 US populations, and were more distant to other sympatric *Pueraria* species. In this study, the clear difference among the five species confirms that ISSR could discriminate closely related *Pueraria* species. The close relationship between the kudzu in the US and

Table 2 Summary of AMOVA results

Taxon	Percentage among group variation = 100 * Φ_{ST} (df)	Percentage within group variation (df)	Probability of no difference among groups (*P*-value)
P. lobata from China and USA	65.4 (1)	34.6 (106)	<0.001
P. lobata from USA	18.7 (18)	81.3 (62)	<0.001
P. lobata from China	74.6 (1)	25.4 (25)	<0.001
P. montana	37.8 (4)	62.2 (70)	<0.001
P. thomsoni	35.1 (2)	64.9 (29)	<0.001

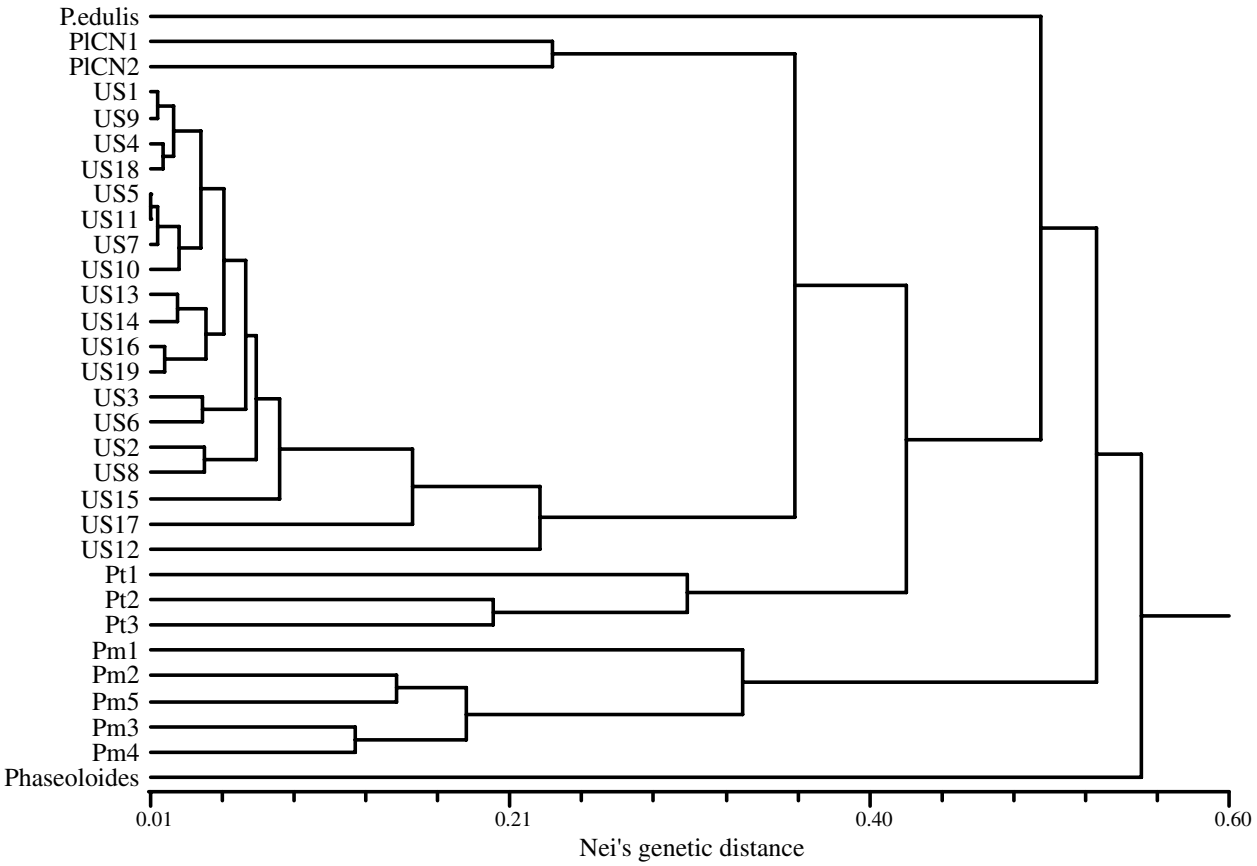

Fig. 1 UPGMA dendrogram based on Nei's (1972) genetic distance. Population identifiers are given in Table 1.

Chinese *P. lobata* indicates that efforts for finding biological control agents should be focused within *P. lobata* in China. *P. montana* and *P. thomsoni*, although morphologically similar, should not be considered as a primary source for control agents and must be properly identified and separated, to avoid including them in any such future studies.

Genetic structure of P. lobata*,* P. montana *and* P. thomsoni *in China*

High genetic differentiation among populations was revealed for the three *Pueraria* species in China, with Φ_{ST} values of 0.746, 0.351 and 0.378 for *P. lobata*, *P. montana* and *P. thomsoni* respectively (Table 2). The low number of populations in each species probably contributed to high Φ_{ST} values, especially in Chinese *P. lobata*. In their review of estimates of genetic diversity obtained with RAPD markers, Nybom and Bartish (2000) compiled mean AMOVA-derived Φ_{ST} values of 0.70, 0.28 and 0.27 for selfing, mixed mating and outcrossing plant species respectively. Thus, genetic differentiation in the three *Pueraria* species was higher than expected for plants with a mixed breeding system. Although Mes (1953) concluded that cross-pollination is necessary for seed setting in *P. lobata*, a certain degree of selfing caused by assortative mating (mating between relatives or partial selfing) cannot be ruled out (Pappert *et al.*, 2000). Possible inbreeding may result in extraordinarily high diversity among populations and low

diversity within populations. Restricted seed dispersal in *Pueraria* (Abramovitz, 1983) might also limit gene flow among populations, thereby increasing their genetic differentiation (Nybom & Bartish, 2000). In addition, vegetative reproduction (Forseth & Teramura, 1986) can help maintain a population primarily by the growth of rhizomes, thus limiting genetic diversity of local populations, while amplifying genetic substructure among populations (McLellan *et al*., 1997).

Genetic diversity of P. lobata *in the US*

Low genetic differentiation (18.7%) was found among the 19 US populations. When the US material was compared with Chinese *P. lobata* populations, AMOVAS showed that there was a 65.4% genetic variation between the US and China, suggesting that genetic differentiation occurred primarily between the two countries. Shannon diversity indices (H_o) of *P. lobata* for Chinese and US samples were 0.208 and 0.221, respectively, indicating similar amounts of diversity. This finding is in contrast to most of the cases of plant colonization. When invasive species colonize a new area, genetic variation is often lower than in the source population, because of frequent founder effects (Tsutsui *et al*., 2000; Sakai *et al*., 2001). For example, introduced populations of *Rubus alceifolius* and *Alliaria petiolata* are genetically less diverse than native populations (Amsellem *et al*., 2000; Meekins *et al*., 2001). Similar results have been reported for other colonizing species (e.g. Novak & Mack, 1993; Young & Murray, 2000).

In this study, because the initial sampling purpose in the USA was not a population genetics study, many populations were represented by very few individuals (one to four; Table 1). The genetic differentiation among populations is thus certainly over-estimated. Indeed, US populations 5, 7 and 11, which have the highest number of individuals, were the closest related over the US group. However, the low number of plants per population did not probably influence the estimation of genetic variation in the US as a whole. A similar genetic structure was reported for the south-eastern USA, *P. lobata* populations based on allozyme markers (G_{ST}: 0.199; Pappert *et al*., 2000). The low level of genetic differentiation among populations in the US was clearly indicated by the low difference among US populations 5, 7 and 11, compared with the high level of genetic structure among Chinese populations of each species (Fig. 1) when numbers of individuals were similar.

Comparison of the genetic diversity in the introduced and the native range of populations may provide valuable information about the process of invasion and explain the observed features (Sakai *et al*., 2001). The number of introduction events is an important factor influencing genetic diversity within the range of an introduced, invasive species (Meekins *et al*., 2001). Species that have entered and quickly spread into a new area via a small number of introduction events may show lower genetic diversity in the introduced range, e.g. *Bromus tectorum* and *Abutilon theophrasti* (Warwick & Black, 1986; Novak & Mack, 1993), because of founder effects, selection and genetic drift. Multiple introductions were suggested to be the 'rule and not the exception' for many introduced plants (Novak & Mack, 1993) and may be the case for *P. lobata* in the US. If plant populations were intentionally introduced many times from different sources, with subsequent gene exchange among different regions or sources, high genetic diversity and low genetic differentiation is expected, such as in the introduced invasive species *Lathyrus latifolius* (Godt & Hamrick, 1991) and *Lonicera japonica* (Schierenbeck *et al*., 1995). *P. lobata* was brought to the US from Japan in the late 1800s. After that, it was repeatedly introduced into the south-eastern US through the late 19th century and the first half of the 20th century, being widely planted to limit soil erosion. Seed and plant multiplication in Soil Conservation Service nurseries certainly both supplemented genetic variation and accelerated gene exchange between different sources. This could have caused the observed genetic diversity of *P. lobata* in the US, as already suggested by Pappert *et al*. (2000). Alternately, the high genetic differentiation between the Chinese and US samples could be due to the limited population samples of *P. lobata* from China, or also to the Japanese origin of US introductions, which was not tested in our study.

Multiple introductions from different sources, followed by subsequent gene exchange and recombination, suggest future challenges for the control of *P. lobata* in the US. *P. lobata* may not uniformly respond to a particular potential biocontrol agent. The high population differentiation of *P. lobata* in China implies that different populations may have different levels of susceptibility or resistance to a pathogen or other biocontrol agents. As *P. lobata* in the US had multiple introductions, classical biological control using natural enemies from its native range may prove to be more complicated than previously thought. At least, consideration should be given to surveying large areas in China and Japan in order to select appropriate potential natural enemies.

Acknowledgements

This is an on-going cooperative project between the United States and China on kudzu biocontrol funded by the USDA Forest Service, Southern Research Station,

Forest Health Protection Enterprise Team and International Programs. The publication of this paper is partially supported by the CAS Key Knowledge Innovation Program (KSCX1-SW-13, KSCX2-SW-104). We are very grateful to Dr Gary DeBarr for initiating this cooperative program. We thank Drs Jim Hanula, Judith Hough-Goldstein and Yong-Ming Yuan for their constructive criticism of the manuscript.

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