Identity of naturalised exotic *Wisteria* (Fabaceae) in the south-eastern United States

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Summary

Exotic *Wisteria* are increasingly serious invasive plants of watersheds and managed forests throughout the south-eastern United States. Naturalised individuals are frequently identified as either *Wisteria floribunda* or *W. sinensis,* but may differ significantly from the original descriptions of either of those species. Here, we use data from the nuclear and chloroplast genomes to determine the species classification or hybrid status of naturalised plants collected in five south-eastern states. Twenty-four of twenty-five collections (96%) were identified as hybrids between *W. floribunda* and *W. sinensis*. Haplotype analyses show that naturalised hybrid *Wisteria* is genetically diverse and that no relationship between haplotype and collection location exists. Morphological characters that clearly differentiate the introduced species cannot be used to reliably identify naturalised individuals. These data, along with observations of the continued spread of *Wisteria* in the south-eastern United States, suggest that hybridisation may be playing a key role in the ongoing invasion of this taxon.

Keywords: invasive plant, genetic variation, diversity, Leguminosae, sequence characterised amplified region, *Wisteria floribunda, Wisteria sinensis*.

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Introduction

Identifying the biological and ecological factors that permit an exotic species to become invasive is one of the basic questions of invasion biology (Elton, 1958; Enserink, 1999). Using a variety of methodologies, investigators have attempted to predict which species may become invasive by comparing their reproductive biology, behaviour in non-native habitats, phenotypic plasticity and taxonomy to known invasive species (e.g. Rejmánek & Richardson, 1996). Within this framework, plants that have a number of shared traits with known invaders would be considered more likely to become invasive.

One basic criticism of these methodologies is that they consider invasive species as immutable entities with fixed characteristics. In fact, the success of the invader may depend more on its ability to respond to natural selection than to a specific biological or ecological characteristic (Lee, 2002). There is growing evidence that suggests invasives are not always 'born', but also arise through various evolutionary mechanisms (Ellstrand & Schierenbeck, 2000). Acquiring knowledge of the genetic makeup of an invasive species is key to determining the events that have led to a species' invasion. Here, we use molecular genetic techniques to examine the identity and possible hybrid nature of naturalised *Wisteria* in the south-eastern United States, and describe how such information may be useful in developing strategies to control further spread.

Wisteria*: a model system*

Chinese (*Wisteria sinensis* (Sims) Sweet) and Japanese *Wisteria* (*Wisteria floribunda* (Willd.) DC) are long-lived perennial vines in the family Fabaceae. Valued for their

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large, pendent racemes of fragrant blue-violet flowers, both species were introduced into the United States in the early 1800s as ornamentals and continue to be important in the horticulture trade (Remaly, 1999).

Individual *Wisteria* vines can live for well over 100 years and can be propagated from both cuttings and seed (Martin, 2002). Their vigorous growth and regeneration capability have been implicated in their escape into native habitats in the United States. Naturalised plants occur throughout the eastern United States, ranging from Vermont to Florida and westward to Texas and Arkansas (USDA NRCS, 2004; Fig. 1). *Wisteria* colonise forest edges, disturbed areas and riparian zones. It grows best in full sun, but is shade tolerant and can be acclimated to a variety of soil and moisture types. *Wisteria* vines are known to strangle or shade out native trees and shrubs, forming dense thickets with few or no other species present (Remaly, 1999). Because of their widespread naturalisation and adverse effect on native habitats, Chinese and Japanese *Wisteria* are considered invasive in 15 eastern states of the United States (Alien Plant Working Group, 2005). The species are listed as invasive by the state Exotic Pest Plant Councils in Georgia, Florida, Tennessee, South Carolina, Virginia (Miller *et al*., 2004; Alabama Invasive Pest Plant Council, 2006) and recently in Alabama. In South Carolina, they are tied as the fifth most common exotic species found in forested habitats (Oswalt, 2006). In west-central Georgia, Lowenstein and Lowenstein (2005) found that exotic *Wisteria* were the seventh most common introduced plants in riparian areas. The fecundity, ease of vegetative propagation and wide range of exploitable habitat all contribute to the aggressive spread of exotic *Wisteria* and suggest a large potential impact in the United States. Subsequently, exotic *Wisteria* are designated and monitored as suspected invasive species by the United States Fish and Wildlife Service (USDA Forest Service, Southern Region, 2001).

The genus *Wisteria* Nuttall includes four currently recognised species (Valder, 1995; Isely, 1998), a number that may grow with taxonomic revision (Zhi, 2005). In the United States, three species commonly occur: the native *W. frutescens* (L.) Poir. (including *W. macrostachya* (Torr. & Gray) Nutt.) and two exotics, *W. floribunda* and *W. sinensis* (Valder, 1995; Isely, 1998). The fourth

Fig. 1 Map of states containing naturalised exotic *Wisteria* as invasive in the United States. Black circles in inset map represent locations of collections used in this study.

Table 1 Morphological key to *Wisteria* species in the United States (adapted from Valder, 1995)

1. Twining anticlockwise	2
1. Twining clockwise	4
2. Standard, ovary and pods glabrous	*W. frutescens*
2. Standard, ovary and pods pubescent	3
3. Pubescence confined to upper third of inner face of standard; leaflets becoming almost glabrous; racemes 12–35 cm; inflorescence buds in winter 4–5 × 2–3 mm	*W. sinensis*
3. Pubescence extending to the base of inner face of standard; leaflets densely pubescent; racemes 12–18 cm; inflorescence buds in winter 12–17 × 5–7 mm	*W. brachybotrys*
4. Pubescence confined to upper third or less of inner face of standard; racemes up to 50 cm; inflorescence buds in winter 5–7 × 2–2.5 mm	*W. floribunda*
4. Pubescence extending to base of inner face of standard; racemes 14–20 cm; inflorescence buds in winter 6–8 × 5–7 mm	*W. brachybotrys* 'Murisaki Kapitan'

species, *W. brachybotrys* Seibold & Zucc., native to Japan, is available commercially. The direction of twining, the number of leaflets and the presence of fruit pubescence are the most commonly used morphological characteristics to differentiate these species (Table 1). These species do not overlap in their home ranges and the morphological characters work well. In contrast, naturalised populations in the United States commonly have 11–13 leaflets and pubescent fruit, but can twine either clockwise or anticlockwise. The plants that twine anticlockwise are usually identified as *W. sinensis,* while those that twine clockwise are identified as *W. floribunda*. Recently, Isely (1998) and Miller (2003) suggested that taxonomic distinction of some naturalised populations may be difficult, due to possible hybridisation between the two species. Although the range of the US native species overlaps with the naturalised populations, they do not hybridise with the exotic species, which is likely due to a difference in ploidy (Valder, 1995; Trusty *et al*., in press).

Materials and methods

Plant tissue was collected from eight naturalised *Wisteria* populations in Alabama, Florida, Georgia, North Carolina and South Carolina during the summer and autumn of 2005 (Fig. 1). Naturalised *Wisteria* were defined as individuals growing along suburban/rural roadsides, local parks and other unplanted locations. Leaf material from two populations of naturalised *Wisteria* was obtained from herbarium specimens through the John D. Freeman Herbarium at Auburn University (AUA; Appendix 1). A reference collection of *Wisteria* species was made from wild-collected plants housed in botanical gardens and from several named horticultural introductions obtained from private collections. Additional details concerning plant material, collection localities, GenBank accession numbers and voucher data are listed in Appendix 1. DNA was extracted from fresh or dried leaf samples using the CTAB protocol (Doyle & Doyle, 1987). These DNA samples were used to amplify species-specific genetic markers from both nuclear and chloroplast DNA.

In order to discover variable DNA regions that were consistent between wild-collected *W. floribunda* and *W. sinensis* individuals, sequence characterised amplified regions were developed from two intersimple sequence repeat primers. Each species was represented by a minimum of three wild collected or horticulturally available samples (data not shown). Primers (CA)6-RY and (CT)8-RA were used under the following PCR reaction conditions: 0.4 μM primer, 1X Taq polymerase buffer, 0.2 μM dNTPs, 0. 25 U Taq polymerase (Eppendorf, Hamburg, Germany) and 1 μL of DNA in a 25 μL reaction volume. Thermocycling conditions were as described by Wolfe *et al*. (1998). Five microlitres of the reaction volume was visualised on a 1% agarose gel containing ethidium bromide in 1X sodium borate buffer. Multiple PCR products of differing sizes were observed. In order to separate each size fragment from the mixture, 1 μL of the PCR product was cloned for each species using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Colonies were screened for inserted fragments using the M13 F and M13R vector primers. PCR products of clones containing fragments larger than 400 bp were cleaned using Microcon PCR filter units (Millipore, Billerica, MA, USA) and sequenced in both directions with plasmid-specific primers using the dideoxy chain termination method with ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA). Sequences

were obtained by electrophoresis on the ABI 3100 automated sequencer in the Auburn University Genomics and Sequencing Lab. Cloned sequences of similar sizes (within 20 bp) from each of the two species were aligned manually and variable homologous nuclear DNA regions were identified.

Nuclear data

Two nuclear regions discovered in the process above, 824 and 997 were amplified separately using primers w898-824F (CATGTTGCATTCAATCTTGG), w898-824R (GCCTCCATACAAGTTAGTTG), w843-997F (GAATCAACGCTGAACGTT), and w843-997AluR (GGTTCAATTTATTGATGTG). These primers were used to amplify all the samples used in this study with the following PCR reaction conditions: 0.4 μM forward primer, 0.4 μM reverse primer, 1X Taq polymerase buffer, 0.2 μM dNTPs, 0. 25 U Taq polymerase (Eppendorf) and 1 μL of DNA in a 25 μL volume. Thermocycler conditions were 94°C for 1 min, 35 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 1 min, followed by 72°C for 10 min.

Region 824—One microlitre of the PCR product was cloned for each sample using the Invitrogen TOPO-TA cloning kit with plasmid vector pCR2, according to the manufacturer's protocol. Colonies were screened for inserts using PCR primers and the thermocycler program described in the cloning kit. PCR products of clones were cleaned using Microcon PCR filter units. Clones were sequenced in two directions with the cloning primers using the dideoxy chain termination method with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit. Sequences were obtained by the Auburn University Genomics and Sequencing Lab on an ABI 3100 automated sequencer following the manufacturer's instructions. Two to four clones were sequenced in two directions for each sample, in order to recover both haplotypes in heterozygous individuals.

Cloned sequences of region 824 were aligned manually across the total sequence length of 654 nt (nucleotides). Each sample had either one (homozygous) or two (heterozygous) haplotypes corresponding to a diploid chromosome number for *Wisteria*. Haplotypes are named by their collection location and correspond to the data in Appendix 1. Parsimony analysis of nuclear region 824 sequences were performed in PAUP* ver. 4.0b10 (Swofford, 2002). A heuristic search with 1000 random taxon addition replicates was performed to identify islands of equally parsimonious trees (Fig. 2; Maddison, 1991).

Region 997—Two primers were designed (w843-997F: GAATCAACGCTGAACGTT and w843-997AluR: GGTTCAATTTATTGATGTG) to amplify 400 bp of nuclear region 997 for all samples. PCR products contained a single HpyCH4 IV cut site at bp 84 in *W. floribunda* haplotypes. For all individuals, 5 μL of amplified product was cut for 1 h at 37°C in the following conditions: 0.5 μL HpyCH4 IV enzyme (New England Biolabs, Ipswich, MA, USA), 2 μL of 10X NEB buffer 1 and 12.5 μL of water. Twenty microlitres of the reaction volume was run out on a 1.5% agarose gel containing 4 μL ethidium bromide in 1X SB buffer and visualised on a UV transilluminator. Haplotypes were scored as *sinensis* (single, uncut band), *floribunda* (two, smaller cut bands) or hybrid (all three bands).

Chloroplast data

The chloroplast *trn L* gene and *trn L-trn F* intergenic spacer (*trn L/*F) of wild-collected *Wisteria floribunda* (four samples) and *W. sinensis* (two samples) were amplified using the 'C' and 'F' primers according to the protocol described in Taberlet *et al.* (1991). Products were sequenced with the amplification primers as described above. Primers (WistrnLF AGTTGACGACATTTCCTTAC and WistrnLR GGAGTGAATGGTTTGATCAATG) were designed to amplify a 250-bp region that contains a 30-bp deletion in *W. sinensis* taxa. Products for all samples were amplified using the Taberlet *et al.* (1991) protocol and visualised on a 1.5% agarose gel. Bands were scored by size as *floribunda* (F) or *sinensis* (S) (Appendix 1).

Results

Nuclear regions

Twenty-four of twenty-five (96%) naturalised individuals were hybrids of *W. floribunda* and *W. sinensis* (Appendix 1). Twenty individuals were hybrid in at least one of the two nuclear regions, two had contrasting nuclear parentage between the two regions, and two hybrids had a single parent for both nuclear DNA regions and the opposite parent for chloroplast DNA. Of the 24 hybrids, 15 (63%) were homozygous for one species at one nuclear region and heterozygous at the other nuclear region, suggesting they were a backcross or other advanced generation hybrid.

Sequence analysis of nuclear region 824 resulted in three unrooted most parsimonious phylograms (length = 90; CI = 0.90; RI = 0.98). A single unrooted phylogram is shown as there is no prior knowledge as to the evolutionary history between the two species (Fig. 2). Haplotypes from *W. floribunda* and *W. sinensis* are separated into two distinct clades. There are a large

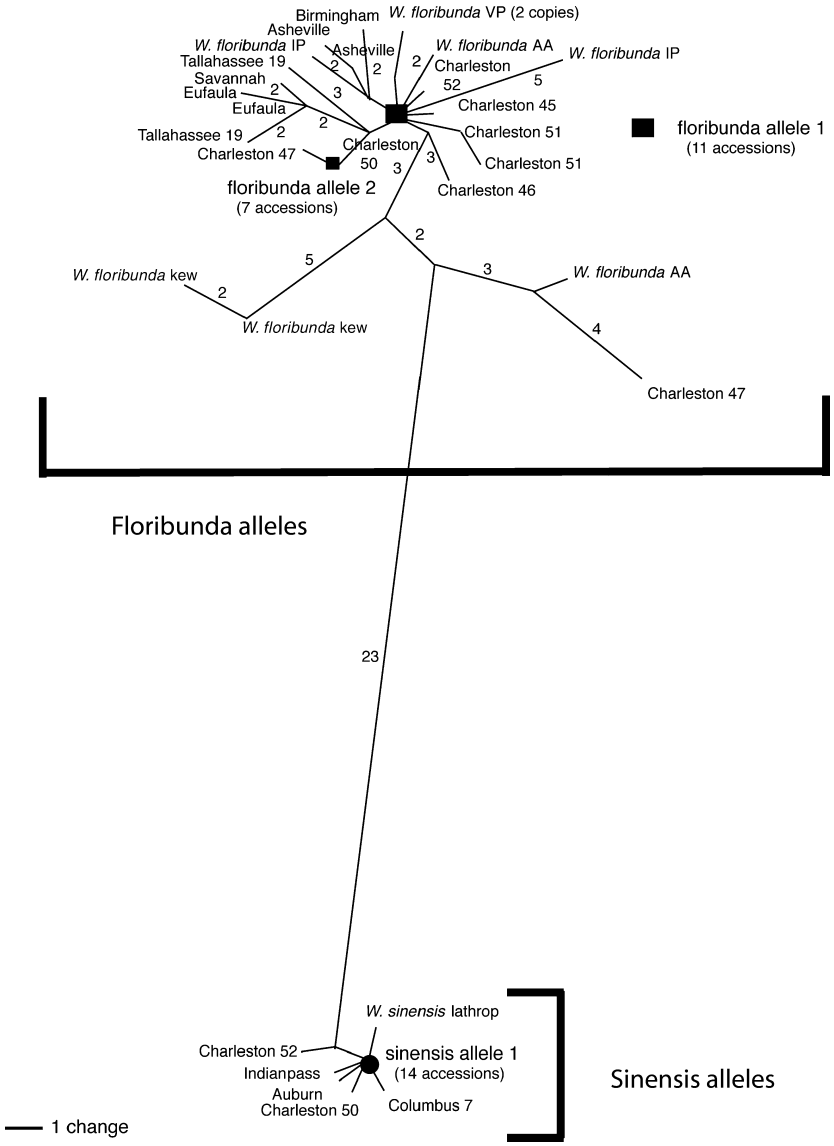

Fig. 2 One of three unrooted most parsimonious phylograms of *Wisteria* dataset. Numbered haplotypes contain multiple accessions and are represented by black rectangles and circle. Accession information for numbered and named haplotypes is listed in Appendix 1. Branch lengths greater than 1 are indicated above each node.

number (>24) of fixed nucleotide substitutions that distinguish the two species.

In total, 35 distinct haplotypes (alleles) were found in 32 individuals. Only six haplotypes were of the *W. sinensis* type, the remaining 29 were *W. floribunda*. In naturalised, hybrid individuals the *W. sinensis* haplotype is frequently identical to the wild-collected *W. sinensis* from China, whereas the *W. floribunda* type haplotypes are much more diverse and distinguishable from the wild-collected haplotypes. The haplotype designation and chloroplast type of all samples is shown in Appendix 1.

The relationships among naturalised haplotypes in either network are not structured by geographical location (Fig. 2). The most common haplotype of *W. floribunda* (found in 10 individuals) occurred in all states sampled, except North Carolina. Similarly, the most common haplotype of *W. sinensis* (found in 12 individuals) was collected in Birmingham and Eufala, AL, Charleston, SC, Columbus, GA, Tallahassee, FL and China.

Chloroplast region

Amplification of the chloroplast *trn* L-F spacer region in this study yielded two easily distinguishable bands differing by 40 nt. No sampled individual was found to be heterozygous (contain both sizes), which suggests uniparental inheritance for this cytoplasmic marker. This chloroplast region was also successfully amplified in 15 wild-collected herbarium samples (13 *W. floribunda* from Japan and two *W. sinensis* from China) and the results corroborated their previous identification. In addition, two of the most recently collected wild *W. floribunda* samples were successfully amplified for nuclear region 997 and were homozygous for floribunda haplotypes as expected (J. Trusty, unpubl. obs.).

Discussion

Exotic plant species have been purposefully introduced by the thousands for food and ornamentation, so, not surprisingly, the majority of invasive plant species emerge from horticultural or agricultural settings (Li *et al*., 2004). In the eastern United States alone, 61–68% of invasive species introduced before 1900 resulted from cultivation for horticultural and medicinal purposes (Mack & Erneberg, 2002). Similarly, horticultural use accounts for more introductions of invasive woody plants in the United States than any other stated purpose (Reichard & White, 2001). Many invasive plants displace native species and associated wildlife and alter natural processes, such as fire and water flow, which further disrupt natural ecosystem function (Cronk & Fuller, 2001). Once established, the removal of invasive species and restoration of natural areas is often difficult and costly (Hiebert, 1997).

Although many plant species are introduced, only a fraction of these species becomes naturalised or subsequently invasive (Williamson & Fitter, 1996). Exotic species are subject to a suite of new environmental and biotic factors, which affect natural selection (Cox, 2005). Only species that are able to respond positively to these new biotic and abiotic regimes naturalise and possibly become invasive. The ability to adapt, and therefore the evolutionary potential and long-term survival of plant species, is directly linked to their genetic diversity (Soltis & Soltis, 1989; Godt & Hamrick, 1991; Richter *et al*., 1994). High genetic diversity arrives with the exotic plant, if the founding population is large enough or if there are multiple introduction events (Rowe *et al*., 1997). In addition, genetic diversity can be gained through hybridisation. For example, many weedy species gain genetic variability by hybridising with closely related native species (Ellstrand & Schierenbeck, 2000). Hybridisation in a species' introduced range also can occur between species or ecotypic races that are geographically isolated in their native range (Daehler & Strong, 1997; Gaskin & Schaal, 2002; Saltonstall, 2002).

Our study has found that nearly all naturalised *Wisteria* sampled are hybrids, and we believe this hybridisation has been a significant step in the evolution of this invasive species. Hybrid *Wisteria* is a cryptic taxon, as hybrid individuals cannot be distinguished by traditional morphological characters, such as the direction of twining or number of leaflets.

Hybrid *Wisteria* carry genetic material encompassing the morphological and ecological traits of both parental species, which probably increases their ability to occupy a novel or wider ecological niche and tolerate ecological extremes (*sensu* Daehler & Strong, 1997; Rieseberg *et al*., 1999). In addition to the genetic potential, hybrids may not have the same susceptibility to herbivores or disease as the parent species (Strauss, 1994; Orians, 2000). Our data indicate that hybrid *Wisteria* is a genetically diverse entity.

Nuclear haplotype data indicate that the most common *Wisteria* haplotypes are shared among sample locations. This would be expected from plants that are propagated and spread rapidly through horticultural means and not through their own movement. If *Wisteria* had colonised the United states from one or a few starting locations, it is likely that genetic differences would have accumulated through time and that there would be a relationship between the genetic diversity and distance between populations. It is possible that hybridisation of the *floribunda* and *sinensis* genomes has occurred many times in different locations or, alternatively, that *Wisteria* hybrids are commonly propagated and spread through horticulture. Understanding how hybrid *Wisteria* has evolved and spread has important implications in understanding its potential for explosive increase in frequency and abundance in forested lands in future.

The obvious presence and growing distribution of *Wisteria* plants in the south-eastern United States suggests that it is already an invasive species. Our data suggest that hybridisation may be playing a key role in its invasiveness. With the hybrid status of naturalised *Wisteria* confirmed, many new avenues for research are opening. Currently, little is known about the reproductive biology of these hybrid swarms, how they are forming and how seeds are being dispersed to new locations. We do not know what, if any, ecological or morphological advantages hybrid plants have. Are they more fecund, grow faster, mature sooner or survive better in disturbed habitats? Finally, knowledge of the timing and location of hybridisation events would help us to understand the dynamics of the population growth and expansion of this invasive species. This information is important in trying to predict the future spread of this species throughout the United States.

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Appendix

Appendix 1 List of plant material used in this study. Pure species outlined in bold. Herbarium voucher locations listed in parentheses

Species	824 nuclear	997 nuclear	Chloroplast	Collection data	GenBank accession numbers
W. floribunda	**F/F**	**F/F**	**F**	**Cultivated at RBG, Kew 2002-1775, wild collected in Japan, Kyoto Pref., Kamigamo**	**EF153219–EF153220**
W. floribunda	**F/F**	**F/F**	**F**	**Cultivated at the Arnold Arboretum 1894-77A; wild collected in Japan**	**EF153221–EF153222**
W. floribunda **'Issai Perfect'**	**F/F**	**F/F**	**F**	**Cultivated by Scott Lathrop, Santa Ana, CA**	**EF153245–EF153246**
W. floribunda **'Violacea Plena'**	**F/F**	**F/F**	**F**	**Cultivated by Scott Lathrop, Santa Ana, CA**	**EF153243–EF153244**
W. sinensis	**S1/S1**	**S/S**	**S**	**Cultivated at RBG, Kew 1985-4607, wild collected in China; Guizhou, near Gyiyang**	**EF153213–EF153214**
W. sinensis	**S1/S**	**S/S**	**S**	**Cultivated by Scott Lathrop, WL074, Santa Ana, CA**	**EF174156–EF174157**
Asheville	F/F	F/S	F	Naturalised, Blue Ridge Parkway at the Swannanoa River, Asheville, Buncombe Co., NC; Trusty 768 (AUA)	EF153225–EF153226
Auburn	F1/S	S/S	S	Naturalised in empty lot; Drake St.; Auburn, Lee Co., AL; Trusty 674 (AUA)	EF153239–EF153240
Birmingham	F/S1	S/S	F	Naturalised; Ruffner Mountain, Jefferson Co., AL; Trusty 767 (AUA)	EF153211–EF153212
Charleston 38	F1/F1	F/S	F	Naturalised, Along West Ashley Greenway, Charleston, Charleston Co., South Carolina; Trusty 751 (AUA)	EF153247–EF153248
Charleston 43	F2/S1	F/S	F	Naturalised, Along West Ashley Greenway, Charleston, Charleston Co., South Carolina; Trusty 756 (AUA)	EF153249–EF153250
Charleston 44	F2/F2	S/S	F	Naturalised, Along West Ashley Greenway, Charleston, Charleston Co., South Carolina; Trusty 757 (AUA)	EF153251–EF153252
Charleston 45	F/S1	F/S	F	Naturalised, Along West Ashley Greenway, Charleston, Charleston Co., South Carolina; Trusty 758 (AUA)	EF153253–EF153254
Charleston 46	F/F1	F/S	S	Naturalised, Along West Ashley Greenway, Charleston, Charleston Co., South Carolina; Trusty 759 (AUA)	EF153261–EF153262
Charleston 47	F/F	S/S	F	Naturalised, Along West Ashley Greenway, Charleston, Charleston Co., South Carolina; Trusty 760 (AUA)	EF153255–EF153256
Charleston 48	F1/S1	S/S	S	Naturalised, Along West Ashley Greenway, Charleston, Charleston Co., South Carolina; Trusty 761 (AUA)	EF153227–EF153228
Charleston 50	F/S	S/S	S	Naturalised, Along West Ashley Greenway, Charleston, Charleston Co., South Carolina; Trusty 763 (AUA)	EF153229–EF153230

Appendix 1 *(Continued)*

Species	824 nuclear	997 nuclear	Chloroplast	Collection data	GenBank accession numbers
Charleston 51	F/F	F/S	F	Naturalised, Along West Ashley Greenway, Charleston, Charleston Co., South Carolina; Trusty 764 (AUA)	EF153231–EF153232
Charleston 52	F1/S	S/S	S	Naturalised, Along US 17, Charleston, Charleston Co., South Carolina; Trusty 765 (AUA)	EF153233–EF153234
Charleston 53	F/S1	S/S	S	Naturalised, Along US 17, Charleston, Charleston Co., South Carolina; Trusty 766 (AUA)	EF153235–EF153236
Columbus 7	S/S1	S/F	S	Naturalised, Hunter Rd., Muscogee Co., GA; Trusty 684 (AUA)	EF153216–EF153217
Columbus 11	F1/S1	S/S	S	Naturalised, Marshall Williams Rd., Harris Co., GA; Trusty 688 (AUA)	EF153218–EF153219
Eufaula	F/F	S/S	S	Naturalised; Hwy 431, Eufaula, Barbour Co., AL Trusty 676 (AUA)	EF153237–EF153238
Indian Pass	F1/S	F/S	S	Naturalised; Along C30A, Indian Pass, Gulf Co., FL; Trusty 675 (AUA)	EF153241–EF153242
Russell County	F1/S1	S/S	S	Naturalised; CR 41, 2 mi. north of AL 169, Russell Co., AL; Gil 2001-311 (AUA)	EF153223–EF153224
Savannah	F/F2	F/F	S	Naturalised, US 17A, Jasper Co., SC; S. Leonard and A. Radford 1241 (AUA)	EF153259–EF153260
Tallahassee 19	F/F	F/F	S	Naturalised, Along Hwy 319, Tallahassee, Leon Co., FL; Trusty 708 (AUA)	EF153201–EF153202
Tallahassee 20	**S1/S1**	**S/S**	**S**	**Naturalised, Along Hwy 319, Tallahassee, Leon Co., FL; Trusty 709 (AUA)**	**EF153203–EF153204**
Tallahassee 22	F1/F2	F/S	F	Naturalised, Along Hwy 319, Tallahassee, Leon Co., FL; Trusty 711 (AUA)	EF153205–EF153206
Tallahassee 23	F2/F2	F/S	F	Naturalised, Along Hwy 319, Thomasville, Thomas Co., GA; Trusty 712 (AUA)	EF153207–EF153208
Tallahassee 24	F1/S1	F/S	S	Naturalised, Along Hwy 319, Thomasville, Thomas Co., GA; Trusty 713 (AUA)	EF153209–EF153210

