

## Research Article

# The Japanese climbing fern (*Lygodium japonicum*) invasion in the U.S.; insights from chloroplast genome sequencing

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## Abstract

Japanese climbing fern (*Lygodium japonicum*) is a vine native to the open forests of eastern Asia that has become an invasive species in the U.S. Herbarium records first noted this species in the U.S. in 1903 (Georgia), with spread to eight states by the end of the 1930s and current establishment in 10 states of the southeastern U.S. We aimed to ask three questions regarding the introduction of *L. japonicum*: (1) Was there a single Japanese climbing fern introduction or were there multiple introductions? (2) What is the distribution of genotypes in the U.S.? and (3) What are the source population(s) from the native range in Asia? We sequenced the chloroplast genome from 74 *L. japonicum* herbarium specimens representing 24 native and 50 invasive range populations. Seventeen haplotypes were found in the native range compared to three in the invasive range. Our results indicate *L. japonicum* has low genotypic diversity in the invasive range relative to the native range. Even with low genotypic diversity, these data suggest at least three introductions of *L. japonicum*. However, we were unable to define the native source population(s) of invasive *L. japonicum*.

**Key words:** Herbarium specimen, invasive species, multiple introductions, southeastern U.S., whole chloroplast genome sequencing



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## Introduction

The process of invasion has the potential to result in reduced genetic variation in introduced populations. If a single or small number of introductions leads to reduced variation, introduced populations may suffer from inbreeding depression and a lack of adaptive potential (Sakai et al. 2001, but see Dlugosch et al. 2015). Most non-native plant species are introduced intentionally for agricultural or aesthetic purposes or unintentionally as contaminants (Mack and Erneberg 2002). Both vectors are expected to be recurrent, which leads to a high probability of multiple introductions. Indeed, single introductions of a plant invasive species are less common than multiple introductions (Dlugosch and Parker 2008). As the number of introductions increases, and admixture occurs between different lineages, novel multi-locus genotypes and increased heterozygosity could increase mean fitness of the invasive (Lee 2002; Colautti and Lau 2015-but see Barker et al. 2019). Uncovering the origin, number of introductions, and genetic diversity of invasive species is important for understanding their history and for their ultimate control (Sakai et al. 2001; Ward et al. 2008). In this study we aim to establish the

number of introductions, source area, and geographic distribution of genotypes in an invasive fern species.

Japanese climbing fern [*Lygodium japonicum* (Thunb.) Sw.] is a vine native to the open forests of East Asia, South Asia, and portions of the East Indies (Hanks 1998). In the U.S. *L. japonicum* has become an invasive species, expanding rapidly in the 20<sup>th</sup> century (Van Loan 2006). Herbarium records first note this species in the U.S. in 1903 (Georgia), with spread to eight states by the end of the 1930s and current establishment in 10 states of the southeastern U.S. (SERNEC Data Portal. 2023). This species is found growing in forests, along waterways, and in disturbed roadside ditches. Japanese climbing fern has twining fronds of indeterminate growth- petioles continue to elongate indefinitely and can reach 90 feet in length (Minogue et al. 2009). This growth pattern allows for a dense canopy that shades out underlying native vegetation. Many reproductive life history characteristics of non-native plants have been proposed to facilitate greater competitive ability and eventual invasion. Such characteristics include self-fertilization, rapid growth to reproductive age, high and continuous seed/spore production, and adaptations for dispersal (Baker 1965). *Lygodium japonicum* possesses a number of these characteristics, combined with a reproductive strategy that presumably allows for rapid geographic spread. Lott et al. (2003) found that more than 90% of isolated *L. japonicum* gametophytes produced successful sporophytes via gametophytic selfing. *Lygodium japonicum* sporangia each produce 256 spores (Murtaza et al. 2004), and even a single spore could suffice to found a new Japanese climbing fern population. Due to this reproductive potential and its ability to reach above tree canopies, long distance wind dispersal and colonization of *L. japonicum* may be achieved by the successful establishment of single spores.

While considerable research exists on the invasion dynamics and control of the congener Old World climbing fern [*Lygodium microphyllum* (Cav.) R. Br.] (Volin et al. 2004, 2010; Gandiaga et al. 2009; Humphreys et al. 2017; David et al. 2020) relatively few focus on Japanese climbing fern. Control via prescribed burning is ineffective and may provide a fuel ladder to canopy trees due to *L. japonicum*'s growth patterns (Minogue et al. 2009). Its underground rhizomes also allow for survival and fast regrowth of vegetation following a burn. Herbicide treatment has been shown to offer some promise, although concerns exist regarding damage to native vegetation (reviewed in Minogue et al. 2009; Bohn et al. 2011). Although biocontrol is another option, no such agents have been identified for this species, with all such efforts focused on *L. microphyllum* (Minogue et al. 2009). Due to the current lack of strong control methodology, it is possible that *L. japonicum* will continue to spread and displace native vegetation throughout the southeastern U.S. Importantly, a lack of information regarding *L. japonicum* genetic variation in the U.S. limits current control efforts. Knowledge of genotypic diversity, origin(s), and genotype distribution is important for controlling invasive plants, especially if there is preliminary evidence that control agents have differential success across multiple genotypes (Ward et al. 2008; Gaskin et al. 2011; Darling 2015; Sun et al. 2020).

In this study we aim to determine if there were multiple introductions of Japanese Climbing Fern, and if so, document the distribution of alternative haplotypes throughout the invaded range. We also aim to determine the source population(s) from the native range in Asia. These three aims are accomplished by sequencing

chloroplast genomes from herbarium specimens throughout the distribution of *L. japonicum* in the U.S. and East Asia. Whole chloroplast genomes are easily obtainable from herbarium specimens of a wide age range (Alsos et al. 2020), and are analytically straightforward due to their uniparental inheritance (Gastony and Yatskievych 1992). Additionally, prior studies have shown chloroplast DNA sequence to be useful for identifying the number of introductions and characterizing the amount of genetic variation transported to the introduced range. Gaskin et al. (2005) found 41 different chloroplast haplotypes in *Lepidium draba* L., with 20 of these haplotypes found in the U.S. Hufbauer and Sforza (2007) examined *Centaurea diffusa* Lam. and *Centaurea stoebe* L. (Asteraceae), and found 11 haplotypes in *Centaurea diffusa* - 9 observed in the native range and only three in the introduced range. The *C. stoebe* sample set contained 11 haplotypes- 10 in the native range, and four in the introduced range (Hufbauer and Sforza 2007). Oduor et al. (2015) observed 32 haplotypes in *Brassica nigra* L., with 22 in the native range and 13 in the introduced range. Williams et al. (2005) observed 10 haplotypes in *Schinus terebinthifolius* G. Raddi- nine in the native range and two in the introduced range. The consistent observation of multiple invasive range haplotypes, albeit fewer than in the native range, suggest that multiple introductions are common but that they are not sufficient to transfer total native-range chloroplast genetic variation to the invasive range. These two observations therefore serve as null expectations for *L. japonicum*.

## Methods

All samples were obtained from herbarium specimens via loans and in-person visits. Each *L. japonicum* herbarium specimen was examined to confirm species identification, and approximately one half of one leaflet was removed and stored in silica gel desiccant. Google Earth Pro (Google Inc. 2021) combined with online place name searches were used to georeference all specimens. DNA extraction was performed using the 96-well protocol presented in Beck et al. (2012). A Qubit model 2.0 fluorometer (Life Technologies, Eugene, Oregon) was used to establish sample DNA concentration with the double-stranded broad-range DNA kit. Samples were chosen for genomic library preparation based on geographic disparity and DNA concentration.

Library preparations were performed using the NEBNext Ultra II DNA Library Prep Kit for Illumina with the NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1) (NEB, Ipswich, Massachusetts). Library preparation followed the protocol outlined in Saeidi et al. (2018). Samples with low library concentrations were re-amplified with universal Illumina primers (Saeidi et al. 2018). Following library preparation, 87 of the 98 samples were selected for sequencing. Unenriched libraries were sequenced with 150 bp paired end chemistry on an Illumina (Illumina, San Diego, California) NextSeq 550 at the University of Kansas Genome Sequencing Core. Trimmed (Trimmomatic- Bolger et al. 2014) chloroplast sequences were then aligned to a published 157,260 bp *Lygodium* chloroplast genome (Genbank accession number KC536645) (Gao et al. 2013) using Geneious (Biomatters, Auckland, New Zealand). Samples exhibiting less than 10,000 reads aligned to the *Lygodium* chloroplast genome were removed, as were four samples with highly divergent chloroplast genomes. These divergent

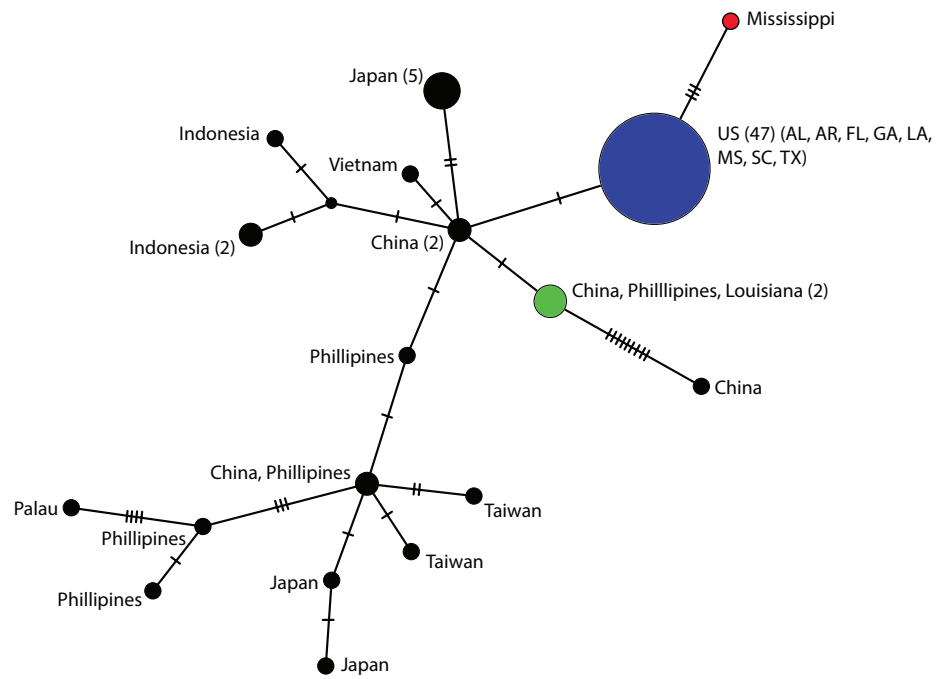
samples also displayed morphologies suggesting they represent other *Lygodium* species. Consensus sequences were formed using a threshold of 75% and nucleotides were called as ambiguous if coverage was less than five. Consensus sequences were aligned using MAFFT (Katoh et al. 2002). Following alignment, all nucleotide positions exhibiting ambiguities, gaps, and identical bases were masked. The average number of pairwise nucleotide differences between samples within both the native and introduced ranges was calculated in DnaSP version 6 (Rozas et al. 2017). A Templeton, Crandall, and Sing (TCS) network (Clement et al. 2002) was produced from the resulting masked alignment in PopArt (Population Analysis with Reticulate Trees) (Leigh and Bryant 2015). The relationship between specimen age and various downstream measures of success (library concentration, mapped reads, coverage) were evaluated with linear regression in R version 4.3.0 (R Core Team 2023).

## Results

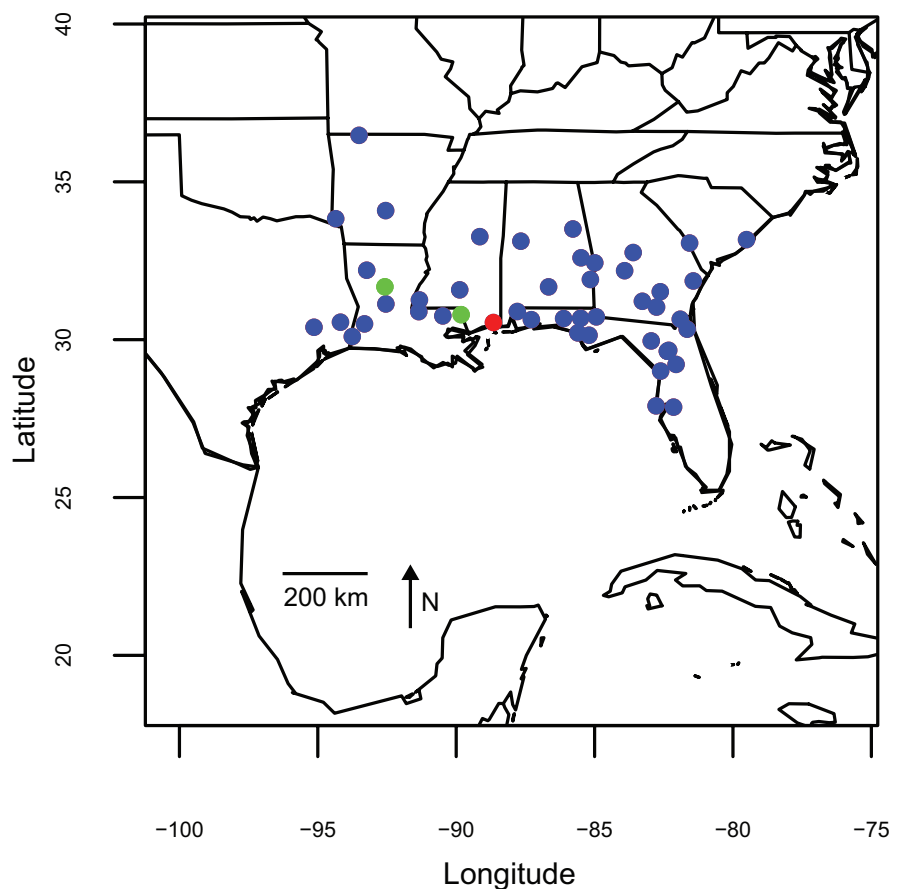
Of the 191 specimens selected for extraction, 189 yielded a measurable DNA concentration. DNA concentration ranged from 0 to 247 ng/ $\mu$ l (mean = 37.64 ng/ $\mu$ l,  $\pm$  41.08 ng/ $\mu$ l). The collection year of extracted specimens ranged from 1910–2017 (mean = 35.05 years old,  $\pm$  15.53 years old). DNA was successfully extracted from both the oldest specimen (137 years old), as well as the youngest specimen (3 years old). After selecting a subset of DNA extractions based on geographic disparity and DNA concentration, 87 samples were chosen for genomic library preparation and sequencing. Library concentration ranged from 1–22.6 ng/ $\mu$ l (mean = 6.08 ng/ $\mu$ l,  $\pm$  3.92 ng/ $\mu$ l), and the relationship between library concentration and specimen age was not significant ( $R^2 = 0.00258$ ;  $p = 0.640$ ).

All sequencing reads are archived on the NCBI Sequence Read Archive (SRA) (BioProject ID #PRJNA1114707). The number of reads mapped to the reference ranged from 800–806,545 (mean = 174,398  $\pm$  166,618). Mean coverage ranged from 0.81–679.77 (mean = 129.74  $\pm$  134.85). Specimen age had a strong negative effect on both the number of mapped reads ( $R^2 = 0.189$ ;  $p = 2.59e^{-05}$ ) and mean coverage ( $R^2 = 0.218$ ;  $p = 5.03e^{-06}$ ). Even with this effect of specimen age, most samples yielded useable chloroplast assemblies, with 85 of 87 samples passing our threshold of >10,000 reads aligned to the *Lygodium* chloroplast genome. Consensus sequences of 74 *L. japonicum* samples with fewer than 10,000 ambiguities were aligned. These consensus sequences included 50 *L. japonicum* samples from the introduced U.S. range and 24 *L. japonicum* samples from the native Asian range (See Suppl. material 1).

Following removal of all ambiguities, gaps, and identical bases from the alignment, 35 SNPs remained. The TCS network (Fig. 1) exhibited 18 *L. japonicum* haplotypes. Sixteen haplotypes were found in the native range, with three present in the introduced range. A single haplotype dominated the invasion and was observed in 47/50 (94%) of U.S. specimens (present in all eight states sampled). It is also the only haplotype seen in South Carolina, Florida, Alabama, Georgia, Texas and Arkansas. Interestingly, this common haplotype was not observed in the native range. The second invasive haplotype was only found in Mississippi, with the third invasive haplotype present in Louisiana as well as China and the Philippines (Figs 1, 2). The average number of pairwise nucleotide differences between samples was higher in the native (4.812) vs. the introduced range (0.277).



**Figure 1.** *Lygodium japonicum* haplotype network. Circles represent observed haplotypes and are scaled to frequency. Black circles denote haplotype observed in the native range only, colored circles denote haplotypes observed in the invasive range and correspond to those seen in Fig. 2. Tick marks denote nucleotide substitutions.



**Figure 2.** Location of *Lygodium japonicum* invasive haplotypes. Colors match haplotypes shown in Fig. 1.

## Discussion

We draw three basic conclusions from our results: (1) there were at least three introductions of *L. japonicum* into the U.S., (2) Japanese climbing fern exhibits a low diversity invasion, and (3) the native source population(s) of *L. japonicum* remain unknown. Our observation of three distinct invasive-range haplotype establishes that multiple introductions have occurred. The only other scenario that would lead to this diversity would be a single introduction comprising multiple haplotypes. We view this as less likely, given the possibility of a single-spore origin of the initial invasive population. Instead, the dominance of the common invasive haplotype suggests that it arrived first and spread throughout the U.S. before the arrival of the two remaining invasive haplotypes. This is supported by collection dates associated with specimens harboring these haplotypes. The common invasive haplotype was the first to be observed (1940, Florida), with the two less common haplotypes observed later (1965, Mississippi; 1996, Louisiana) (See Suppl. material 1). An early arrival of the common invasive haplotype is, of course, consistent with its geographic dominance, although the seemingly late arrival of the less common haplotypes could simply be due to the likelihood of observing a relatively rare sequence. Inferring multiple introductions is not unexpected, since these have been commonly observed in prior reviews of plant isozyme studies (Dlugosch and Parker 2008) and in a variety of investigations using chloroplast sequence data (McIvor et al. 2001; Gaskin et al. 2005; Williams et al. 2005; Hufbauer and Sforza 2008; Oduor et al. 2015; Holt et al. 2023). Multiple introductions did not, however, lead to high introduced-range genetic diversity. Only three haplotypes were observed in the U.S., one of which was found in 94% of U.S. samples. The dominance of this haplotype and the relatively low genetic distance between any of the three haplotypes found in the U.S. also led to a markedly lower average number of pairwise nucleotide differences within the introduced range relative to the native range. The only published study that has examined whole chloroplast genome diversity in a North American invasion is that of *Salvinia molesta* D. Mitch. (Holt et al. 2023), in which nine unique haplotypes were observed in the U.S. In that study, 21 U.S. samples were sequenced, so a unique haplotype was observed for every two samples sequenced. Three haplotypes were observed in 50 U.S. *L. japonicum* samples (1 for every 17 samples sequenced). Although this relative lack of diversity among the *L. japonicum* genomes we sequenced suggests that this is a low diversity invasion, comparisons to future studies of whole chloroplast genome diversity in U.S. invasions should be considered. Consistent with many other comparisons of native and introduced range chloroplast sequence data (McIvor et al. 2001; Gaskin et al. 2005; Williams et al. 2005; Hufbauer and Sforza 2008; Oduor et al. 2015) this level of diversity (3 haplotypes) was also a clear reduction from that seen in the native range (16 haplotypes).

Regarding native-range source areas, the only haplotype match was between one observed twice in Louisiana to one observed in China and the Philippines (Fig. 1). Although this suggests two possible source populations, the fact that we did not observe a match to the overwhelmingly common introduced haplotype suggests that the geographic source of the bulk of the invasion remains unknown. This lack of a match between the most common introduced-range chloroplast haplotype and a native-range haplotype is unusual, as such a match is frequently observed in similar studies (Saltonstall 2002; Gaskin et al. 2005; Williams et al. 2005;

Hufbauer and Sforza 2008; Oduor et al. 2015). The lack of such a match could be due to insufficient native-range sampling, although it does suggest that the dominant introduced-range haplotype is relatively uncommon in the native range. An alternative explanation for this pattern involves a novel mutation, as the common invasive haplotype is only a single mutation removed from one observed in two Chinese samples (Fig. 1). We view this as unlikely, as such a mutation would have had to have occurred in the very narrow time window between initial introduction and the spread of this now novel haplotype throughout the invasive range.

Knowledge of genotypic diversity, source(s) and genotype distribution is important for controlling invasive plants, especially if there is preliminary evidence that control agents have differential success across multiple genotypes (Charudattan 2005; Gaskin et al. 2005; Morin et al. 2006; Ward et al. 2008; Gaskin et al. 2011; Darling 2015; Sun et al. 2020). Specifically, our finding of low genetic diversity overall and a single haplotype dominating the invasion suggest that a biocontrol solution for *L. japonicum* might be feasible. The strategy for finding a biocontrol agent usually involves searching within the native range of the invasive species for natural enemies that are both host specific and damaging to the invasive species (Roderick and Navajas 2003). In the case of the Japanese climbing fern, work on *L. microphyllum* serves as an example. Goolsby et al. (2004) identified several genotypes of lygodium gall mite (*Floracarus perrepae* Knihinicki & Boczek) from Australia and Asia. They concluded that mites had differing success on the invasive fern genotype; the mite genotypes that performed best came from regions where the native fern genotypes were most similar to the invasive genotype (Goolsby et al. 2004). In the case of *L. japonicum*, the fact of a low diversity invasion therefore potentially simplifies the process of identifying biocontrol genotypes. Ideally, knowing the source location(s) of the *L. japonicum* invasion would greatly narrow the search for appropriate natural enemy genotypes, and further work should expand native range sampling to discover the source region(s) for the common genotype observed in the U.S. invasion. Additionally, similar studies of invasive and native-range genetic diversity should be conducted with high-resolution nuclear data (Peterson et al. 2012). Until biocontrol methods are implemented, it is likely that *L. japonicum* will continue to spread and displace native vegetation throughout the southeastern U.S.

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## Additional information

### Conflict of interest

The authors have declared that no competing interests exist.

### Ethical statement

No ethical statement was reported.

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## Author contributions

All authors have contributed equally.

## Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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## Supplementary material 1

### Sample information

Authors: Morgan L. Markley, Ethan Altergott, James B. Beck

Data type: xlsx

Explanation note: Information for the 74 *L. japonicum* samples used to construct the haplotype network.

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