

## Research Article

**Cite this article:** Tippery NP, Sabol MM, Koehler JG, Topol CE, Crossgrove K (2025) Assessing the genetic composition of invasive knotweeds (*Reynoutria*, Polygonaceae) using data from the first intron and second exon of the nuclear *LEAFY* gene. *Invasive Plant Sci. Manag.* **18**(e1), 1–12. doi: [10.1017/inp.2024.36](https://doi.org/10.1017/inp.2024.36)

Received: 29 May 2024

Revised: 28 August 2024

Accepted: 12 November 2024

### Associate Editor:

Ryan Thum, Montana State University


### Keywords:

Bohemian knotweed; *Fallopia japonica*; giant knotweed; Japanese knotweed; next-generation sequencing; *Polygonum cuspidatum*; *Reynoutria japonica*

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# Assessing the genetic composition of invasive knotweeds (*Reynoutria*, Polygonaceae) using data from the first intron and second exon of the nuclear *LEAFY* gene

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## Abstract

In places where multiple related taxa are invasive and known to hybridize, it is important to have correct identifications to enable an appropriate legal, ecological, and management understanding of each kind of invader. Invasive knotweeds in the genus *Reynoutria* Houtt. are noxious weeds in Europe, North America, Africa, and Oceania, where they disrupt native plant communities and negatively impact human activities. Two species (Japanese knotweed [*Reynoutria japonica* Houtt.; syn.: *Polygonum cuspidatum* Siebold & Zucc.] and giant knotweed [*Reynoutria sachalinensis* (F. Schmidt ex Maxim.) Nakai; syn.: *Polygonum sachalinense* F. Schmidt ex Maxim.]) and their hybrid (known as Bohemian knotweed [*Reynoutria* × *bohemica* Chrtek & Chrtková; syn.: *Polygonum* × *bohemicum* (J. Chrtek & Chrtková) Zika & Jacobson [*cuspidatum* × *sachalinense*]]) have similar invasive tendencies, although there are some noted differences among them in their reproduction potential, ecological tolerance, and effect on native communities. Prior studies demonstrated that not only one kind of interspecific hybrid exists, but in fact there are at least four kinds that differ in the sequence variants they possess from each parent. Thus, in addition to identifying plants as hybrids, it may become important to distinguish each kind of hybrid when considering control or treatment strategies. In the current study, we expand the available genetic information for invasive *Reynoutria* by providing expanded DNA sequence data for the low-copy nuclear gene *LEAFY*, which has become important for characterizing hybrids. Our methods recover the same *LEAFY* genotypes that were identified previously for the commonly sequenced second intron, and we also provide sequence data for the first intron and second exon of the gene.

## Introduction

Plant invasions frequently manifest as two or more congeneric species with similar ecological profiles (Gaskin et al. 2024; Green 1966; Kartesz 2015; Lubell et al. 2008; Maddox et al. 2010). When two invasive congeners grow together, these can hybridize to produce a new invasive taxon (Ellstrand and Schierenbeck 2000; Schierenbeck and Ellstrand 2009; Welles and Ellstrand 2020). It is essential to identify species and hybrids correctly, because the different genetic entities could have different ecological tolerances or responses to control methods (David et al. 2015; Tataridas et al. 2022; Yang et al. 2011). Moreover, the legal status of one taxonomic entity may differ from another, and this discrepancy can delay an effective management response in cases of uncertain identifications (Fox and Gordon 2009; Randall et al. 2008).

Invasive knotweeds in the genus *Reynoutria* Houtt. (syn.: *Polygonum* L.; Polygonaceae) are a growing concern in several parts of the world. Originally restricted to a relatively narrow native distribution in eastern Asia, these plants have become noxious weeds in Europe (Lavoie 2017), North America (Del Tredici 2017), Oceania (Desjardins et al. 2023b), and Africa (Germishuizen 1986). Japanese knotweed [*Reynoutria japonica* Houtt.; syn.: *Polygonum cuspidatum* Siebold & Zucc.], giant knotweed [*Reynoutria sachalinensis* (F. Schmidt ex Maxim.) Nakai; syn.: *Polygonum sachalinense* F. Schmidt ex Maxim.], and their hybrid known as Bohemian knotweed [*Reynoutria* × *bohemica* Chrtek & Chrtková; syn.: *Polygonum* × *bohemicum* (J. Chrtek & Chrtková) Zika & Jacobson [*cuspidatum* × *sachalinense*]]) have similar invasive potential in temperate habitats, owing to their aggressive clonal growth (Bailey et al. 2009), their allelopathic effects on neighboring plants (Murrell et al. 2011; Parepa et al. 2012; Vrchotová and Šerá 2008), and their tenacious underground rhizomes that resist eradication (Drazan 2022; Lawson et al. 2021). The negative impacts of invasive *Reynoutria* species include ecosystem disruption

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### Management Implications

Invasive knotweeds (*Reynoutria* spp.) pose a threat to ecosystems across Europe and North America. There are numerous genetic entities in the invaded range, including two species (*Reynoutria japonica* [Japanese knotweed] and *Reynoutria sachalinensis* [giant knotweed]) and several distinct kinds of hybrids (collectively known as Bohemian knotweed [*Reynoutria*  $\times$  *bohemica*]). The different genetic entities vary with respect to attributes that affect their invasiveness, including reproductive strategies, growth rate, and competitive ability. It benefits land managers to know the genetic identities of their invasive *Reynoutria* plants so that they can develop effective strategies for prevention and control. In this study, we expand on previous work and provide additional data regarding the genetic makeup of invasive *Reynoutria*. We present novel methods for identifying plant material, including a next-generation sequencing method and a cost-effective method that uses polymerase chain reaction (PCR). The newly developed methods should enable rapid and confident identification of invasive *Reynoutria* plants.

(Drazan et al. 2021; Gerber et al. 2008; Lecerf et al. 2007; Maerz et al. 2005; Serniak et al. 2017; Siemens and Blossey 2007) and a variety of negative consequences for humans (McLean 2010).

Although these *Reynoutria* species and hybrids are all considered invasive outside their native range, differences in their ecological attributes may impact their mechanism of spread, habitat tolerances, competitive abilities, and susceptibility to control methods. For example, the hybrid *R.  $\times$ bohemica* has been shown to grow more rapidly and inhibit the growth of native species more strongly than either *R. japonica* or *R. sachalinensis* (Bímová et al. 2003; Mandák et al. 2004; Moravcová et al. 2011; Parepa et al. 2014). Control methods often are applied indiscriminately to invasive *Reynoutria* regardless of taxon, but there can be some value to using taxon-specific management approaches (Camargo et al. 2022; Clements et al. 2016; Kadlecová et al. 2022; Yoshimoto and Szűcs 2024). Therefore, it would be valuable to identify which taxon (or taxa) is present before attempting to manage it (Bailey et al. 2007). Although morphological characters exist for differentiating the various invasive *Reynoutria* taxa (Zika and Jacobson 2003), molecular methods offer an independent method for improving confidence and confirming the identification of morphologically intermediate plants (Tippery et al. 2021). Fortunately, molecular methods can identify invasive *Reynoutria*, including hybrids, using DNA sequence data from individual genes (Park et al. 2018; Tippery et al. 2021) or from a wide range of variable sites across the genome (Drazan 2022).

*LEAFY* (*LFY* or *LFY3*, also known as *FLORICAULA/FLO*; *Arabidopsis* AT5G61850; Berardini et al. 2015) is a low-copy nuclear gene that encodes a transcriptional regulator involved in floral meristem development (Frohlich and Meyerowitz 1997; Gao et al. 2019; Moyroud et al. 2009). Nuclear gene sequences can provide biparentally inherited information for reconstructing phylogenetic relationships, and more importantly, such genes can be useful for distinguishing parental species and their allopolyploid descendants. *LEAFY* has been used to infer the parental species involved in generating allopolyploid species in *Fagopyrum* Mill. and *Persicaria* Mill. (Kim et al. 2008; Nishimoto et al. 2003) as well as Polygonaceae more broadly (Sanchez and Kron 2008). In recent

years, the utility of *LEAFY* has expanded substantially to include plants from across the angiosperm phylogeny (e.g., Archambault and Bruneau 2004; Grob et al. 2004; Howarth and Baum 2005).

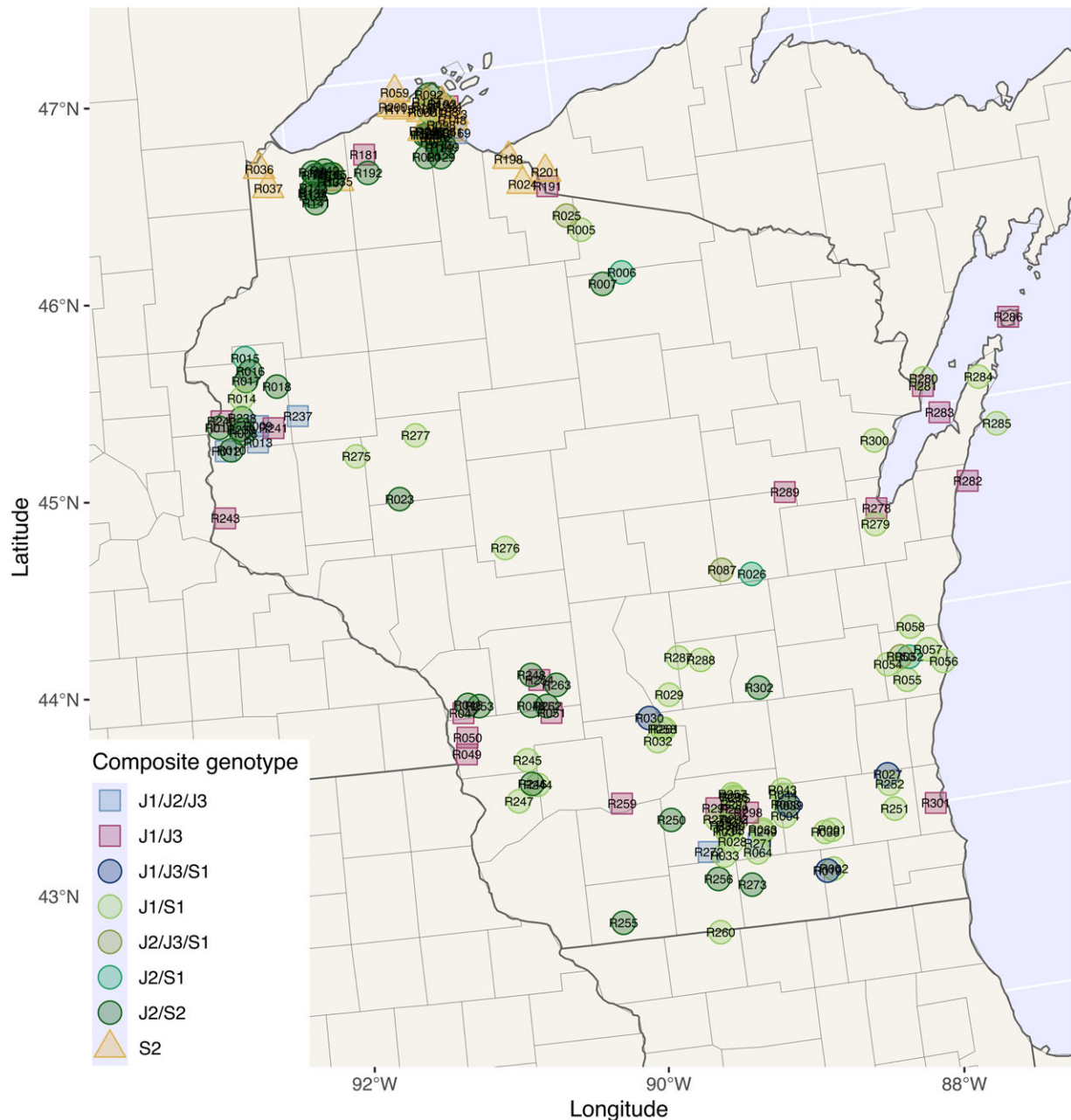
The *LEAFY* gene structure is conserved across angiosperms and consists of three exons and two introns (Weigel et al. 1992). Phylogenetic studies have relied upon the second intron, which can be reliably sequenced and is sufficiently large and variable for many phylogenetic applications. Prior studies of *Reynoutria* have produced more than 20 unique sequences for the *LEAFY* second intron and provided support for the phylogenetic distinctness of several species: *R. japonica* and *R. sachalinensis*, as well as *Reynoutria compacta* [dwarf knotweed] (Hook.f.) Nakai and *Reynoutria multiflora* [tuber fleecflower] (Thunb.) Moldenke (Desjardins et al. 2023a; Park et al. 2018; Tippery et al. 2021). Analysis of *LEAFY* second intron sequences for invasive *Reynoutria* in the United States recovered four kinds of sequence that were attributed to *R. japonica* and two sequences that were attributed to *R. sachalinensis* (Tippery et al. 2021). The same publication describes how five different *LEAFY* sequence combinations were recovered from plants identified as *R.  $\times$ bohemica*, suggesting a high amount of interbreeding among species and hybrids.

The existing *LEAFY* second intron sequences for *Reynoutria* species have been effective for identifying invasive species and hybrids, and we anticipate that gathering additional sequence data would improve the utility of this region for species identification and phylogenetic analysis. For example, a study in the related genus *Fagopyrum* recovered nearly complete *LEAFY* sequences (including substantial portions of all introns and exons), and these provided some of the first data for *LEAFY* gene structure outside the model organism *Arabidopsis thaliana* (Nishimoto et al. 2003). We endeavored to obtain sequences from the first intron and second exon to learn about the protein-coding portion of the *LEAFY* gene and to compare the amount of variation between the two intron regions. We anticipate that the enhanced dataset will enable more precise and more efficient identifications of *Reynoutria* species and hybrids by providing a larger set of nucleotide polymorphisms that can be compared.

## Materials and Methods

### Morphological Data Collection

Samples of *Reynoutria* species were collected from Wisconsin, USA (Figure 1; Supplementary Table S1) and were targeted to include all genetic variants that had been documented previously (Tippery et al. 2021). Morphological data for lamina base, abaxial vein hairs, inflorescence size, and reproductive condition were recorded from dried specimens, as described previously (Tippery et al. 2021). Additionally, we measured the length of the longest available lamina for each specimen (i.e., located in the distal 20 cm of a branch) as the length from the petiole attachment point to the tip of the lamina apex. Characters were scored numerically as follows: lamina base (0 = truncate without apparent lobes, 0.5 = intermediate with lobes <2 cm, 1 = with lobes >2 cm), abaxial vein hairs (0 = hairs absent or evident only as scabrous protrusions, 0.5 = hairs unicellular and <2-mm long, 1 = hairs multicellular and >2-mm long), inflorescence size (0 = shorter than leaf at the same node, 0.5 = approximately equal, 1 = longer than leaf at the same node), and reproductive condition (0 = male sterile, 1 = male fertile). Lamina length was recorded as a continuous numerical variable.



**Figure 1.** Map of *Reynoutria* collection sites in Wisconsin. Shapes indicate taxonomic identity (square, *R. japonica*; circle, *R. xbohemica*; triangle, *R. sachalinensis*), and colors depict unique composite genotypes. Numbers inside the shapes refer to specimen ID (Supplementary Table S1). Site positions are “jittered” to facilitate viewing adjacent sites, using a random uniform distribution of 0.1° in both latitude and longitude.

### Molecular Data Collection

Primers for polymerase chain reaction (PCR) were developed initially by aligning and comparing sequences of the *LEAFY* gene from a phylogenetic study of *Fagopyrum* (Nishimoto et al. 2003). Subsequent primers were designed using novel sequence data from *Reynoutria* taxa. Ultimately, the following primers were used most effectively: Flint1-F1, Japo8F, and Reyn3F, located in the first exon; Japo5R and Reyn4F, both located in the second exon; and MLFYI2-2385R (Schuster et al. 2011), located in the third exon (Table 1). The plastid *matK* region was amplified using the primers AF and 8R (Yan et al. 2008).

Genomic DNA was extracted using the CTAB method (Doyle and Doyle 1987), modified as described by Tippers et al. (2020). PCR was conducted using the Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA), with a 58 C annealing temperature and 30-s extension time. PCR products were cleaned enzymatically using the *ExoI* and *FastAP* enzymes (Thermo Fisher Scientific). Sanger sequencing (Sanger et al. 1977) used the same primers that were used for PCR reactions and was conducted through Eurofins Genomics (Louisville, KY, USA). In situations where Sanger sequencing produced polymorphic results, separate sequences were obtained either by subcloning PCR



**Table 1.** DNA sequences for oligonucleotide primers that were used to amplify portions of the *LEAFY* gene in *Reynoutria* species.

Name	Sequence	Location	Alignment position <sup>a</sup>	Source
Flint1-F1	5'-RGAGTTATCCARGCTTACGG-3'	Exon 1	1	This study
Japo8F	5'-AACAGCCTCTCTCATATCTTCCG-3'	Exon 1	116	This study
Reyn3F	5'-TGGCCTCAGCCTCATGTCCG-3'	Exon 1	238	This study
Reyn4F	5'-GGGAGAGAAAGAAAGTGGTCCG-3'	Exon 2	849	This study
Japo5R	5'-GTGCAACTTCTCCAGGCTCC-3'	Exon 2	1110	This study
Sach1F	5'-AGCTAGTTAGCTAGGTAGTAC-3'	Intron 2	1325	This study
Sach1R	5'-TTCATATTTGTAGCTACGTAGC-3'	Intron 2	1334	This study
Japo1F	5'-GGTAAAGTGAGAGACGTATAAATG-3'	Intron 2	1338	This study
Japo1R	5'-CATTTATACGTCTCTCACTTACC-3'	Intron 2	1338	This study
MLFYI2-2385R	5'-TGCCTAYCTGAACACTTGGTTYGT-3'	Exon 3	2379	Schuster et al. 2011

<sup>a</sup>Intron 1 is located at alignment positions 299–834, and intron 2 is located at positions 1223–2375.

products into bacterial vectors or by conducting next-generation sequencing. For bacterial subcloning, the PCR products were cleaned using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and cloned into bacteria using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific), then colonies of transformed cells were subjected to PCR and sequencing as above. For next-generation sequencing, PCR products were cleaned using 0.1 volumes of 3 M ammonium acetate and 4 volumes of 100% ethanol, followed by one wash with 80% ethanol. Sequencing was conducted by Eurofins Genomics using Oxford Nanopore Technologies (Oxford, UK).

### Molecular Data Analysis

Newly obtained sequences were combined with previously published sequences (Desjardins et al. 2023a; Park et al. 2018; Tipperty et al. 2021) and aligned manually using Mesquite v. 3.81 (Maddison and Maddison 2023). *LEAFY* sequence variants were attributed to species according to their prior identification (Desjardins et al. 2023a; Park et al. 2018) or their phylogenetic relatedness to previously identified sequences (see “Results and Discussion”). Sequence alignments were analyzed using the APE package v. 5.7.1 (Paradis and Schliep 2009) for R v. 4.4.1 (R Core Team 2024). After model selection using IQ-TREE v. 2.0.5 (Minh et al. 2020; Nguyen et al. 2015), phylogenetic analyses were conducted in BEAST v. 1.10.4 (Suchard et al. 2018) with the GTR+G model of evolution (Tavaré 1986), using 10 million generations of Markov chain Monte Carlo (MCMC) (Hastings 1970; Metropolis et al. 1953), sampled every 5,000 generations, and trees were summarized after the first 25% of trees were discarded as burn-in. Sequence alignment data for the two *LEAFY* introns were evaluated separately and together.

To evaluate distances among novel and previously published sequences effectively, the sequence alignment was trimmed to include only regions having complete data for all nucleotide sequences (i.e., only the *LEAFY* second intron). Insertions/deletions (indels) were coded using simple indel coding (Simmons and Ochoterena 2000) in the program SeqState v. 1.4.1 (Müller 2005). After removal of identical sequences from the data matrix, genetic distances among *LEAFY* gene sequences were visualized using the program SplitsTree v. 6.3.25 (Huson and Bryant 2006) with *p* distances (Hamming 1950) and NeighborNet method (Bryant and Huson 2023; Bryant and Moulton 2004).

Putative amino acid translations of the *LEAFY* exons were generated in Mesquite after aligning *Reynoutria* and *Fagopyrum* sequences and coding equivalent nucleotide positions as codons (Nishimoto et al. 2003). Similarity comparisons were made by

conducting a protein BLAST search of the putative translations (Altschul et al. 1990; Johnson et al. 2008).

### Diagnostic PCR

Primers for diagnostic PCR reactions were developed by identifying regions that consistently differed among types of *Reynoutria* sequences. Four novel primers were developed and named according to whether they amplified *R. japonica* (Japo) or *R. sachalinensis* (Sach) sequence variants. Japo1F or Sach1F was used in combination with MLFYI2-2385R. Japo1R or Sach1R was used with Reyn3F. All diagnostic primers were designed to anneal to locations in the *LEAFY* second intron. Diagnostic PCR reactions were run as described earlier for other primers, and amplicons were run using electrophoresis on a 2% agarose gel containing GelStar™ nucleic acid stain (Lonza Bioscience, Walkersville, MD, USA). PCR products (4 µl of each) were run alongside 2 µl of GeneRuler 1 kb Plus DNA ladder (Thermo Fisher Scientific).

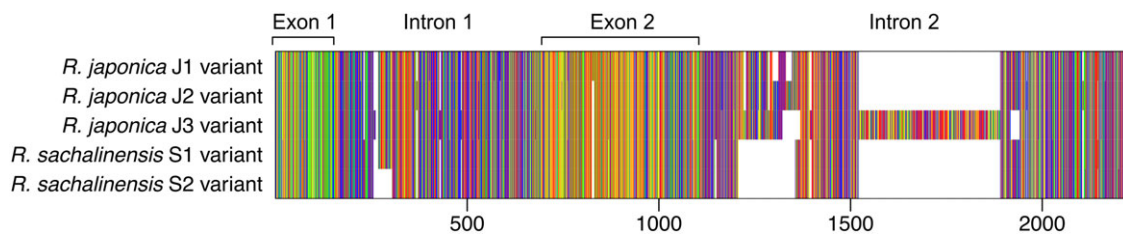
### Morphological Analyses

Morphological data were evaluated for individuals with known *LEAFY* gene sequences. Data were visualized using the GGPlot2 package v. 3.5.1 in R (R Core Team 2024; Wickham 2016). Plants were grouped by their composite genetic makeup (i.e., all types of *LEAFY* sequence variants that were recovered for each individual), and the differences among genetic variants were evaluated using ANOVA (Fisher 1921). Specifically, we used the *aov* function in the STATS package, followed by the Tukey's test (Tukey 1949) via the *HSD.test* function in the AGRICOLAE package v. 1.3.7 in R (Mendiburu 2021; R Core Team 2024). Principal component analysis (Pearson 1901) was done via the *prcomp* function in the R STATS package (R Core Team 2024) using the variables of lamina length, lamina base, and abaxial vein hairs, after removing individuals that lacked data for one or more of the variables.

## Results and Discussion

### Molecular Data Summary

Sequence data from the *LEAFY* gene were obtained for 156 *Reynoutria* specimens (Figure 1; Supplementary Table S1). Five unique sequence variants were identified, and these were assigned codes that corresponded to previously identified variants (Tipperty et al. 2021). (It remains unclear whether any of the *LEAFY* sequence variants are inherited as alleles for homologous chromosomes, and therefore we avoid referring to sequence variants as “alleles.” We refer to the collection of all sequence



**Figure 2.** Alignment of *LEAFY* sequences for invasive *Reynoutria* taxa, showing the most commonly encountered sequences for each sequence variant. Sequences are identified as originating from *R. japonica* (J1/J2/J3) or *R. sachalinensis* (S1/S2). Intron and exon borders are indicated; the third exon is not shown but would appear shortly to the right of the last nucleotide shown.

variants obtained for a single individual as its “composite genotype.”) Three sequence variants were attributable to *R. japonica* (J1, J2, J3), and two variants were attributed to *R. sachalinensis* (S1, S2) (Park et al. 2018; Tippery et al. 2021).

Up to three variants were recovered from any one individual. Thirty-two accessions contained only *R. japonica* sequence variants (composite genotypes J1/J3 and J1/J2/J3), and 20 accessions contained only *R. sachalinensis* sequence variants, specifically the S2 variant. The remaining plants all contained a combination of sequence variants from both species, which is consistent with their identification as hybrids. There was minimal variation among sequences that were identified herein as corresponding to the same sequence variant (Supplementary Figures S1 and S2).

Sequence alignment for the *LEAFY* second intron was 1,149 nucleotides in length and included 26 polymorphic sites and 42 indels (Figure 2). The first intron was 483 to 528 nucleotides in length and included 19 polymorphic sites and 10 indels. The second exon was 402 to 408 nucleotides in length and included 5 polymorphic sites and 1 indel. The second intron was 599 to 1,056 nucleotides in length and included 27 polymorphic sites and 14 indels. The second intron of the J3 sequence variant included a 369-nucleotide insertion relative to the other variants (Figure 2). Sequence data for the first exon were incomplete but comprised 240 nucleotides, of which 3 sites were polymorphic, with no indels. Because of the location of the MLFY12-2385R primer (Schuster et al. 2011), we obtained only a negligible amount of sequence data for the third exon.

### Molecular Data Analyses

Phylogenetic analysis of *LEAFY* sequences recovered two clades that corresponded to *R. japonica* and *R. sachalinensis*, respectively (Figure 3; Supplementary Figures S1 and S2). Newly reported sequence variants frequently were most similar to previously reported sequences from invasive plants in New Zealand or the United Kingdom. For example, our J2 variant closely matched accession ON586877 from New Zealand, the J3 variant matched ON586876 from New Zealand, and both S1 and S2 were very similar to JF831231 from the United Kingdom. Notably, the J1 variant did not share close similarity with any previously published sequences from outside the United States. The phylogenetic position of *R. compacta* relative to *R. japonica* and *R. sachalinensis* was poorly supported and inconclusive.

Translation of the protein-coding regions of the *LEAFY* gene produced amino acid sequences that were comparable with equivalent regions in *Fagopyrum*. Protein BLAST of the translated coding region from the longest sequence among the novel accessions (R046: 213 amino acids) returned a maximum sequence identity of 80.1% with *Fagopyrum macrocarpum* [largeseed

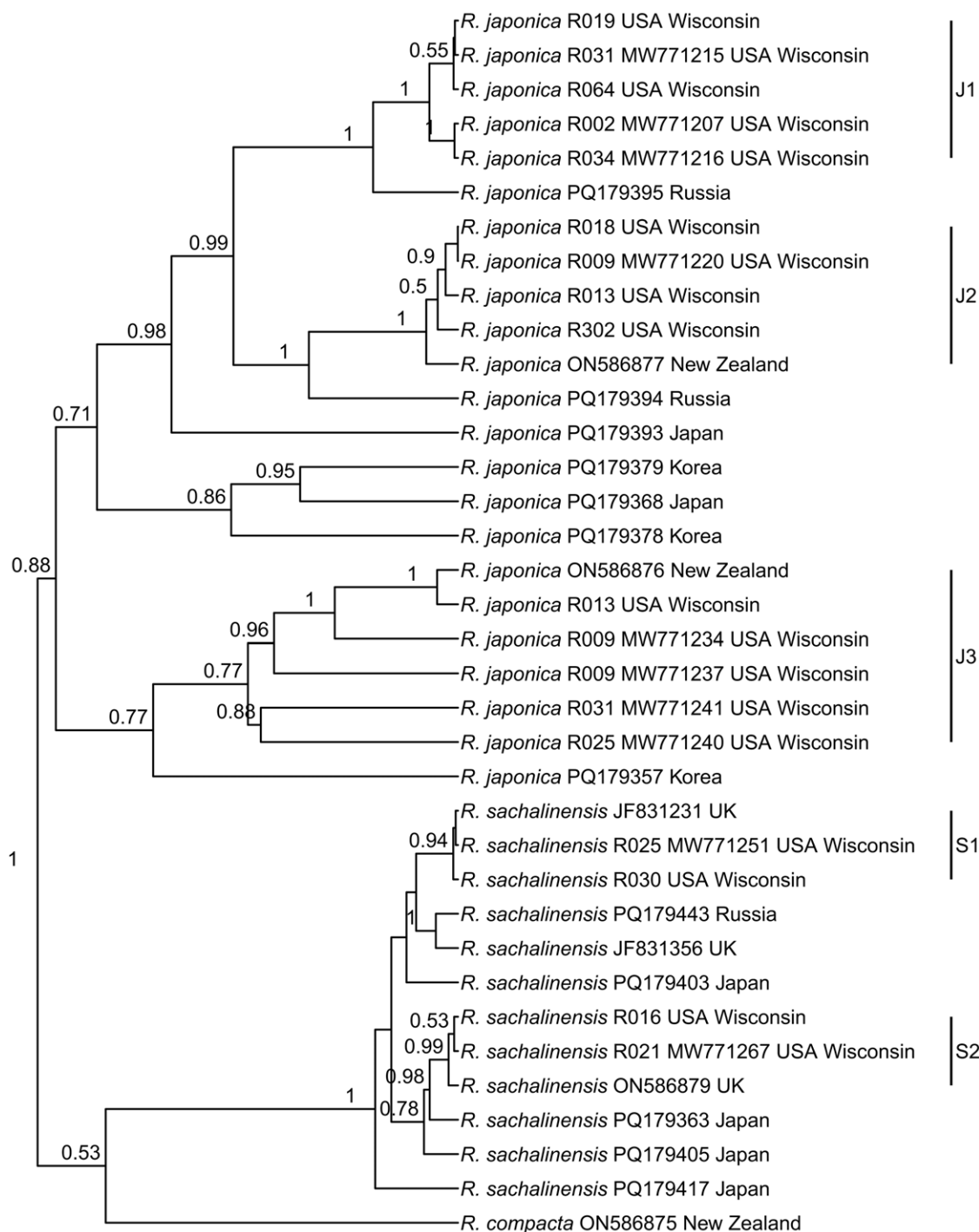
buckwheat] Ohsako & Ohnishi (GenBank accession no. BAC76911). Comparison of translated *LEAFY* gene sequences among *Reynoutria* taxa showed that two amino acid positions were consistently different between sequences identified as *R. japonica* or *R. sachalinensis*. Additionally, there was an indel, two amino acids in length, in a region that consisted of either four or six glutamine residues, in a region that also was variable among *Fagopyrum* sequences (Nishimoto et al. 2003).

Data from the plastid *matK* region placed most of the accessions into the clade corresponding to *R. japonica*, except for accessions that had only *R. sachalinensis* *LEAFY* sequences (with no evidence of hybridization), in which case the latter group had *matK* sequences that matched *R. sachalinensis* (data not shown). The fact that plastid sequences for hybrids match *R. japonica* is consistent with that species being the original maternal parent of all hybrids, and this also is consistent with the evidence that *R. japonica* is male-sterile throughout its invasive range (Forman and Kesseli 2003; Grimsby et al. 2007; Hollingsworth and Bailey 2000; Tippery et al. 2021).

The SplitsTree network for *LEAFY* sequences separated *R. japonica* sequence variants from those of *R. sachalinensis* (Figure 4). The shortest distance between sequence variants was between S1 and S2 of *R. sachalinensis*. The J3 variant of *R. japonica* showed the greatest dissimilarity among sequences assigned to the same variant. The *LEAFY* sequence for *R. compacta* was most similar to the J1 sequence variant of *R. japonica*, from which it differed by 13 changes. Sequence variants for *R. japonica* sequences were separated from *R. sachalinensis* sequences by a minimum of 12 sequence differences. The maximum distance between any 2 *R. japonica* sequence variants was 30 differences, and the maximum distance between *R. sachalinensis* sequence variants was 5 differences.

Diagnostic PCR reactions corroborated the genotype evidence that was obtained using DNA sequencing (Figure 5). The expected lengths of PCR products were as follows: Japo1F/MLFY12-2385R: 638 to 678 bp; Sach1F/MLFY12-2385R: 548 to 549 bp; Reyn3F/Japo1R: 1,102 to 1,106 bp; Reyn3F/Sach1R: 1,055 to 1,091 bp. Individuals that had only *R. japonica* sequence variants failed to amplify products using the Sach1F or Sach1R primers, individuals with only *R. sachalinensis* sequence variants failed to produce products using Japo1F or Japo1R, and all diagnostic primer pairs produced PCR products for individuals whose genetic makeup included sequence variants from both species (Figure 5). A variety of hybrid genotypes were tested, including individuals for each of the single-species genotypes as well as multiple kinds of hybrids.

This study investigated the feasibility and diagnostic value of gathering additional sequence data from the *LEAFY* first intron and second exon, regions that have seldom been sequenced in angiosperms. We were able to obtain sequence data from these regions for all previously identified genetic variants in the United

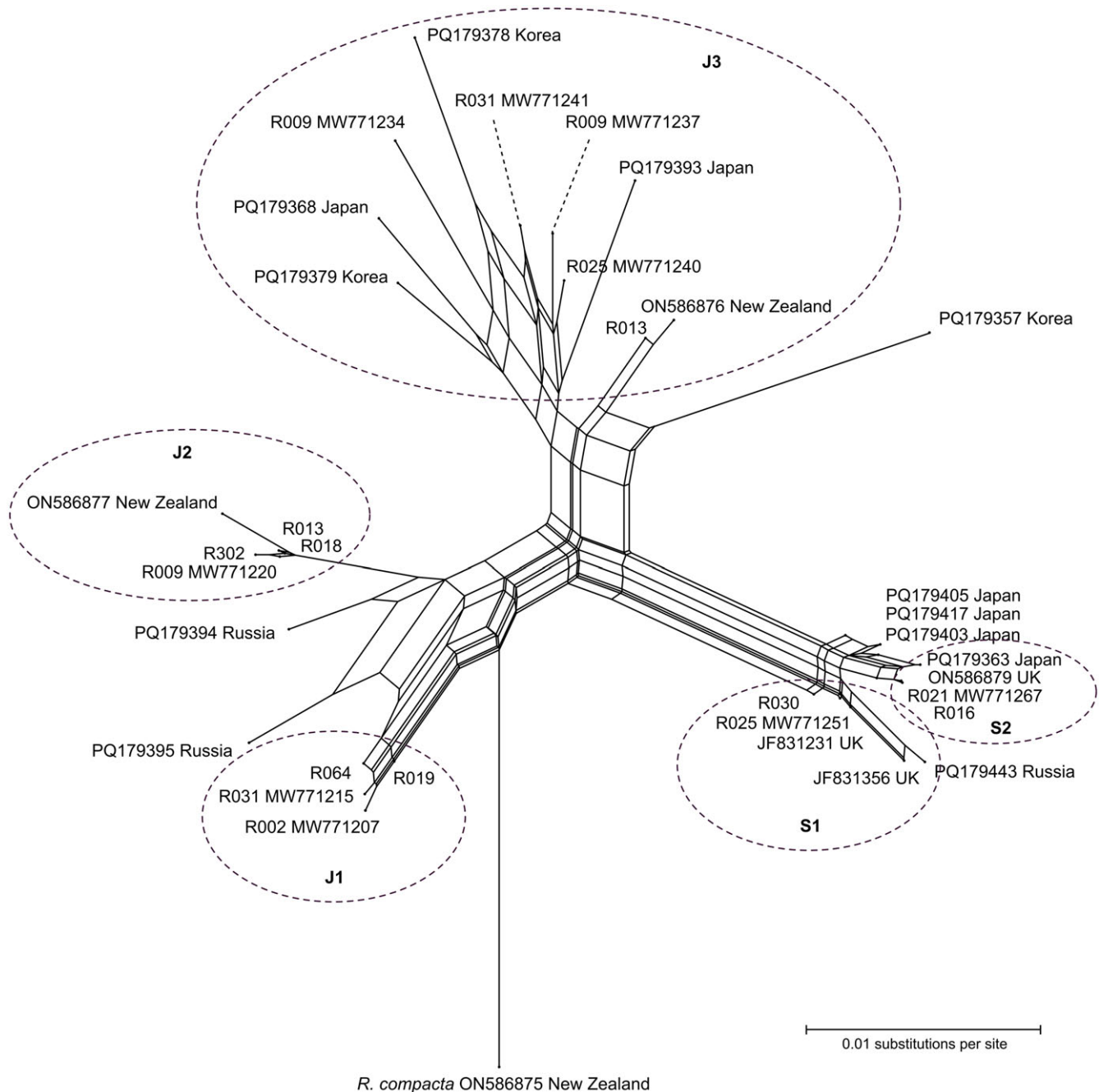


**Figure 3.** Phylogenetic relationships among sequences obtained from *Reynoutria* species. Sequences are identified to species according to their previous identifications (Desjardins et al. 2023a; Park et al. 2018; Schuster et al. 2011) or by their similarity to previously published sequences. Previously published sequences are labeled with GenBank accession numbers. Sequences from plants in Wisconsin (Tippery et al. 2021; this study) have a unique identifier (e.g., R019) that is referenced in Supplementary Table S1. Sequence variant names (e.g., J1) are given for plants collected from the invasive range. Nodal support values indicate posterior probability; values less than 0.5 are not shown.

States, with the result that about twice as many nucleotides (relative to prior studies) could be evaluated to assess genetic variability and phylogenetic relationships. We developed novel PCR primers that could be used to sequence additional *Reynoutria* taxa as well as species in related genera. Currently, there are no comparable first intron or second intron sequences for plants in the native range of *Reynoutria* or other portions of the invaded range, and these would be valuable to compare.

### Morphological Data Summary

We obtained morphological data for the same *Reynoutria* accessions that were used to extract molecular data (Figure 1; Supplementary Table S1). There were 60 specimens with complete morphological data for all characters evaluated, whereas the remaining specimens either lacked reproductive material or had lost all flowers to abscission. Inflorescence



**Figure 4.** SplitsTree network of *Reynoutria* LEAFY sequences. Each label represents a unique sequence variant that is connected to similar sequences by lines. Previously published sequences are identified with GenBank accession numbers. Sequences from plants in Wisconsin (Tipperty et al. 2021; this study) have a unique identifier (e.g., R019) that is referenced in Supplementary Table S1. Sequence variant names (e.g., J1) are given for plants collected from the invasive range.

length data were recorded for 93 specimens. Lamina base morphology and abaxial vein hair morphology were recorded for all 156 specimens. Lamina length across all specimens (all species) ranged from 8 to 29 cm.

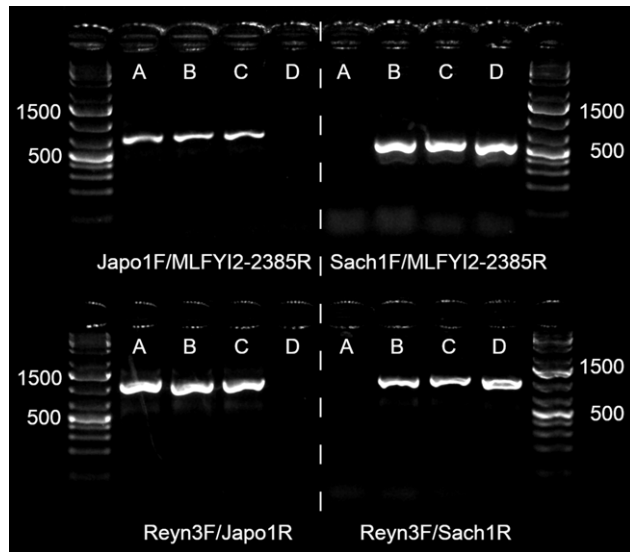
### Morphological Data Analyses

Our extensive sampling of *Reynoutria* plants in Wisconsin has enabled us to evaluate the relationship between composite genotype and morphology. It has been established previously that hybrid *R. ×bohemica* plants have intermediate morphological characteristics (Tipperty et al. 2021; Zika and Jacobson 2003), but

the morphologies of various hybrids have not been explored in light of their genetic differences. Genetically variable hybrids may have morphological traits that resemble those of one or the other parental species (Jordon-Thaden et al. 2023; Mitchell et al. 2022; Rieseberg et al. 2003). Thus, it is important to evaluate morphological differences among *Reynoutria* plants that have different genomic contributions from *R. japonica* and *R. sachalinensis*.

Plants in our study with only *R. japonica* sequence variants (J1/J2/J3 and J1/J3 composite genotypes) mostly exhibited the expected morphology for that species: truncate lamina base, absent abaxial vein hairs, and male-sterile flowers (Figure 6). Long abaxial





**Figure 5.** Results of diagnostic polymerase chain reaction (PCR) using primers that selectively amplify either *Reynoutria japonica* or *Reynoutria sachalinensis* sequence variants. The top row shows the result of two primer pairs, separated by a dotted line, both using the MLFYI2-2385R reverse primer located in the *LEAFY* third exon, with a discriminant forward primer located in the second intron. The bottom row shows the result of two primer pairs, separated by a dotted line, and both using the Reyn3F forward primer located in the *LEAFY* first exon, with a discriminant reverse primer located in the second intron. The same source DNAs were used for all four PCR reactions: (A) R012, (B) R039, (C) R026, and (D) R071 (Supplementary Table S1). R012 has only *R. japonica* sequence variants, R071 has only *R. sachalinensis* sequence variants, and the remaining two DNAs contain sequence variants from both species. Two sizes (in bp) are labeled for the size standards, corresponding to the brightest bands at those locations.

vein hairs were observed in most (but not all) specimens having the S2 composite genotype and rarely in other composite genotypes, whereas intermediate vein hairs were found occasionally in several composite genotypes (Figure 6C). Inflorescence length was expected to be longer than leaf length for *R. japonica*, but in fact very few specimens of any genotype had long inflorescences (Figure 6D). Plants with only *R. sachalinensis* sequence variants (S2 composite genotype) mostly had the expected traits of long basal lamina lobes, multicellular abaxial vein hairs, and male-fertile flowers.

Among the hybrids, basal lamina lobes >2 cm were rarely encountered, except in the J2/S2 composite genotype (Figure 6B). Notably, this was the only hybrid composite genotype with an S2 sequence variant. Male-sterile plants were rarely encountered among hybrids (Figure 6E). Lamina length was widely variable overall (Figure 6A), yet *R. sachalinensis* plants and plants with the J2/S1 or J2/S2 composite genotypes had significantly longer laminae than *R. japonica* plants (one-way ANOVA:  $F(7, 143) = 22.37$ ,  $P < 2 \times 10^{-16}$ ). The J1/S1 and J2/S2 hybrids had a significantly different mean for their lamina base morphology (Figure 6B), but it should be noted that both truncate bases and lobes of intermediate size were present in both kinds of hybrids. The lamina base trait was evaluated using dried herbarium specimens, and it may be possible to gain a clearer understanding of the differences by using more precise measurements on fresh leaves.

Importantly, the morphological analysis revealed that some hybrid individuals with the J1/S1 or J2/S2 composite genotypes are indistinguishable from non-hybrid *R. sachalinensis* plants, and

other individuals with these same composite genotypes were not different from *R. japonica* plants. Principal component (PC) analysis (Figure 6F) separated plants on the PC1 axis mostly by lamina base, and the other two morphological traits contributed strongly to the PC2 dimension. Plants largely clustered by composite genotype, and overall the two most commonly encountered hybrid genotypes (J1/S1 and J2/S2) were poorly differentiated from each other. Nonetheless, the hybrids largely could be distinguished from *R. japonica* by having longer laminae and from *R. sachalinensis* by lacking basal lamina lobes and multicellular abaxial vein hairs.

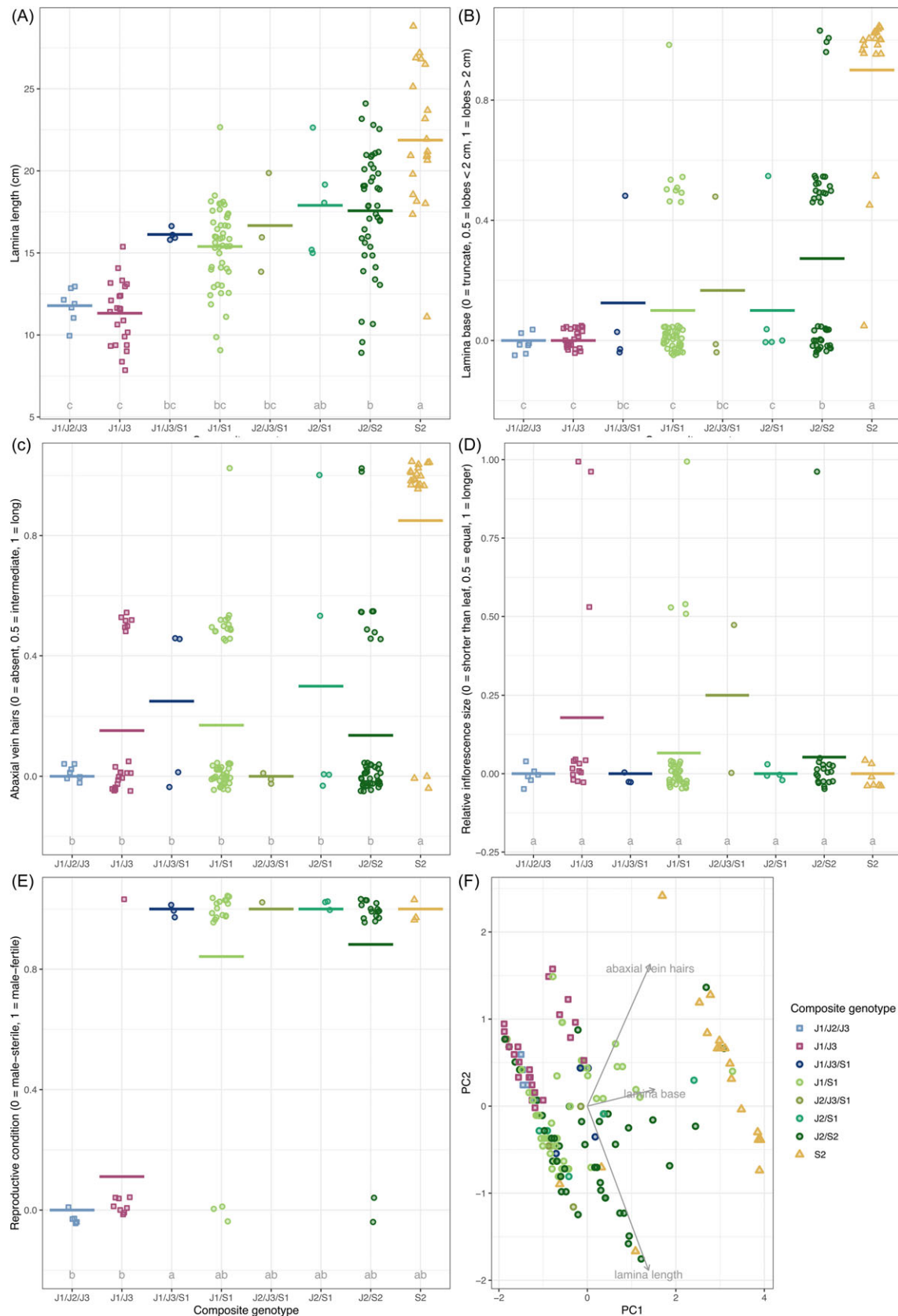
### Widespread Hybridization

The high similarity between *LEAFY* sequences for *R. japonica* and *R. sachalinensis* (Figures 2–4) supports prior evidence that these species are closely related and occasionally hybridize in their native range (Park et al. 2018; Tippery et al. 2021). Hybrid genotypes appear prominently in the invaded range, where they are associated with measurably different invasiveness patterns (Bimová et al. 2003; Mandák et al. 2004; Moravcová et al. 2011). Our study corroborates prior evidence that the invasive U.S. *Reynoutria* hybrids comprise at least five distinct genotypic combinations, presumably the result of separate original hybridization events (Tippery et al. 2021).

The existence of multiple versions of the *LEAFY* gene in some *Reynoutria* individuals likely reflects polyploidy in the genus, which has been documented in the native and invasive ranges (Iwatsubo et al. 2004; Kim and Park 2000; Mandák et al. 2003). In Europe, different ploidy levels characterize each taxon, with octaploid *R. japonica* and tetraploid *R. sachalinensis* having hybridized to produce the predominantly hexaploid *R. xbohemica* (Bailey et al. 2007; Te Beest et al. 2012), although a variety of ploidy levels were observed for the latter taxon (Saad et al. 2011). We recovered no more than three sequence variants from any one individual, and the only taxon we encountered with one variant per individual was *R. sachalinensis* (containing the S2 sequence variant only). The genetically diverse array of hybrids has yet to be correlated with ploidy levels, and a chromosomal investigation may produce insights into the origins of hybrid genotypes. We maintain that using a single hybrid name, *R. xbohemica*, may hinder a more nuanced understanding of hybridization in *Reynoutria*, and we support efforts to distinguish hybrids by their genetic composition rather than simply by their parent species (Tippery et al. 2021).

Four of the five *LEAFY* gene variants we identified were very similar to sequences that were recovered from invasive plants in New Zealand and the United Kingdom (Figures 3 and 4). Invasive species frequently are genetically similar across their invasive ranges (Benoit et al. 2019; Tippery et al. 2023), resulting in part from introduction bottlenecks and anthropogenic movement (Dlugosch and Parker 2008; Smith et al. 2020). Only the J1 variant from our study lacked a comparable sequence from outside the United States, and this could indicate that the variant is only present in U.S. invasive plants or that the collection and molecular analysis protocols that have been used thus far in other countries have failed to locate a plant with the J1 variant. We recommend that molecular tools should be used across the invasive range to determine the genetic similarity among invasive *Reynoutria* plants worldwide and potentially to reconstruct the origins of various composite hybrid genotypes. Moreover, it may be possible to





**Figure 6.** Morphological data for *Reynoutria* plants with known composite genotypes. In each panel, shapes correspond to taxonomic identity (square, *R. japonica*; circle, *R. × bohemica*; triangle, *R. sachalinensis*), and colors depict unique composite genotypes. Positions are “jittered” by a value of 0.2 to facilitate viewing adjacent points. The first five panels each show morphological data for one trait: (A) lamina length, (B) lamina base, (C) abaxial vein hairs, (D) relative inflorescence size, and (E) reproductive condition. The final panel (F) shows the principal component (PC) analysis, with vectors showing the relative contributions of the three morphological traits that were evaluated.

ascertain whether hybridization continues to generate novel genetic combinations.

Comparisons among amino acid sequences from the second exon, both within *Reynoutria* and in the related genus *Fagopyrum*, are consistent with the possibility that all versions of *LEAFY* could be functional. At this point we have not obtained evidence to suggest that any sequence variants have become nonfunctional, as can happen in polyploids (Adams and Wendel 2005; Edger and Pires 2009; Roulin et al. 2013). Currently, *Fagopyrum* is the most phylogenetically similar genus whose *LEAFY* protein sequence can be compared with that of *Reynoutria*, and it would be beneficial to obtain equivalent sequences from the more closely related genera *Fallopia* Adans. or *Muehlenbeckia* Meisn. (Schuster et al. 2011), as well as other *Reynoutria* species.

The term “next-generation sequencing” describes a variety of methods that can produce sequences from individual fragments of sample DNA (Goodwin et al. 2016). For heterogeneous pools of DNA, such methods can efficiently separate out allelic variants (e.g., Macas et al. 2011). Thus, next-generation sequencing offers an alternative to bacterial subcloning, which also can be effective for isolating DNA sequence variants (e.g., Moody and Les 2002). In this study, we employed both next-generation sequencing and bacterial subcloning and obtained equivalent results. We found the next-generation sequencing method to be cost-effective and more rapid than bacterial subcloning, and we recommend this method as a viable strategy for future plant identifications.

Several diagnostic primers were tested, and we confirmed their effectiveness for identifying plants that have *R. japonica* and/or *R. sachalinensis* sequence variants. Conducting PCR, followed by gel electrophoresis (and sometimes also including restriction enzyme digest), is a faster and less expensive alternative to DNA sequencing (e.g., Saltonstall 2003; Wendell et al. 2021). The diagnostic primers reported herein are able to identify plants as species or hybrids; however, they were not designed to distinguish specific sequence variants from each species. The newly reported *LEAFY* gene sequences also enable additional diagnostic options to be explored, such as variant-specific sequencing primers (Scheen et al. 2012) or diagnostic primers for environmental DNA (eDNA) analysis (e.g., Kuehne et al. 2020).

The invasive *Reynoutria* are now well represented by *LEAFY* sequence data that include the first intron and second exon. However, there are other *Reynoutria* taxa, as well as species in related genera, for which no *LEAFY* sequences have been published. The methods employed herein should be applied to other taxa to assess phylogenetic relationships and investigate recent hybridization or polyploid species origins.

**Supplementary material.** To view supplementary material for this article, please visit <https://doi.org/10.1017/inp.2024.36>

**Data availability.** Newly generated DNA sequences were deposited to GenBank, under the accession numbers that are referenced in Supplementary Table S1.

**Acknowledgments.** We are grateful for the assistance of plant collectors and the coordinators of the plant collecting efforts, in particular Maureen Kalschauer (Wisconsin Department of Natural Resources) and Matt Wallrath (University of Wisconsin Extension). We appreciate the comments and suggestions of two anonymous reviewers that helped us to improve the article.

**Funding statement.** Funding was provided by the University of Wisconsin–Whitewater Undergraduate Research Program and the University of Wisconsin–Whitewater Department of Biology.

**Competing interests.** The authors declare no conflict of interest.

## References

- Adams KL, Wendel JF (2005) Polyploidy and genome evolution in plants. *Curr Opin Plant Biol* 8:135–141
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410
- Archambault A, Bruneau A (2004) Phylogenetic utility of the *LEAFY/FLORICAULA* gene in the Caesalpinioideae (Leguminosae): gene duplication and a novel insertion. *Syst Bot* 29:609–626
- Bailey JP, Bímová K, Mandák B (2007) The potential role of polyploidy and hybridisation in the further evolution of the highly invasive *Fallopia* taxa in Europe. *Ecol Res* 22:920–928
- Bailey JP, Bímová K, Mandák B (2009) Asexual spread versus sexual reproduction and evolution in Japanese knotweed *s.l.* sets the stage for the “battle of the clones.” *Biol Invasions* 11:1189–1203
- Benoit LK, Les DH, King UM, Na HR, Chen L, Tippery NP (2019) Extensive interlineage hybridization in the predominantly clonal *Hydrilla verticillata*. *Am J Bot* 106:1622–1637
- Berardini TZ, Reiser L, Li D, Mezheritsky Y, Muller R, Strait E, Huala E (2015) The *Arabidopsis* information resource: making and mining the “gold standard” annotated reference plant genome. *Genesis* 53:474–485
- Bímová K, Mandák B, Pyšek P (2003) Experimental study of vegetative regeneration in four invasive *Reynoutria* taxa (Polygonaceae). *Plant Ecol* 166:1–11
- Bryant D, Huson DH (2023) NeighborNet: improved algorithms and implementation. *Front Bioinform* 3:1178600
- Bryant D, Moulton V (2004) Neighbor-net: an agglomerative method for the construction of phylogenetic networks. *Mol Biol Evol* 21:255–265
- Camargo AM, Kurose D, Post MJ, Lommen ST (2022) A new population of the biocontrol agent *Aphalara itadori* performs best on the hybrid host *Reynoutria × bohemica*. *Biol Control* 174:105007
- Clements DR, Larsen T, Grenz J (2016) Knotweed management strategies in North America with the advent of widespread hybrid Bohemian knotweed, regional differences, and the potential for biocontrol via the psyllid *Aphalara itadori* Shinji. *Invasive Plant Sci Manag* 9:60–70
- David AS, Zarnetske PL, Hacker SD, Ruggiero P, Biel RG, Seabloom EW (2015) Invasive congeners differ in successional impacts across space and time. *PLoS One* 10:e0117283
- Del Tredici P (2017) The introduction of Japanese knotweed, *Reynoutria japonica*, into North America. *J Torrey Bot Soc* 144:406–416
- Desjardins SD, Bailey JP, Zhang B, Zhao K, Schwarzhacher T (2023a) New insights into the phylogenetic relationships of Japanese knotweed (*Reynoutria japonica*) and allied taxa in subtribe Reynoutriinae (Polygonaceae). *PhytoKeys* 220:83
- Desjardins SD, Pashley CH, Bailey JP (2023b) A taxonomic, cytological and genetic survey of Japanese knotweed *s.l.* in New Zealand indicates multiple secondary introductions from Europe and a direct introduction from Japan. *NZ J Bot* 61:49–66
- Glugosch KM, Parker IM (2008) Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. *Mol Ecol* 17:431–449
- Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull* 19:11–15
- Drazan D (2022) Genetic Diversity, Structure, and Cold Hardiness of Invasive Knotweed (*Fallopia* spp.) in Minnesota. MS thesis. Twin Cities: University of Minnesota. 166 p
- Drazan D, Smith AG, Anderson NO, Becker R, Clark M (2021) History of knotweed (*Fallopia* spp.) invasiveness. *Weed Sci* 69:617–623
- Edger PP, Pires JC (2009) Gene and genome duplications: the impact of dosage-sensitivity on the fate of nuclear genes. *Chromosome Res* 17:699–717
- Ellstrand NC, Schierenbeck KA (2000) Hybridization as a stimulus for the evolution of invasiveness in plants? *Proc Natl Acad Sci USA* 97:7043–7050
- Fisher RA (1921) Studies in crop variation. I. An examination of the yield of dressed grain from Broadbalk. *J Agric Sci* 11:107–135

- Forman J, Kesseli RV (2003) Sexual reproduction in the invasive species *Fallopia japonica* (Polygonaceae). *Am J Bot* 90:586–592
- Fox AM, Gordon DR (2009) Approaches for assessing the status of nonnative plants: a comparative analysis. *Invasive Plant Sci Manag* 2:166–184
- Frohlich MW, Meyerowitz EM (1997) The search for flower homeotic gene homologs in basal angiosperms and Gnetales: a potential new source of data on the evolutionary origin of flowers. *Int J Plant Sci* 158:S131–S142
- Gao B, Chen M, Li X, Zhang J (2019). Ancient duplications and grass-specific transposition influenced the evolution of *LEAFY* transcription factor genes. *Commun Biol* 2:1–10
- Gaskin JF, West N, Rector BG (2024) Population structure of three invasive congeneric teasel (*Dipsacus*) species. *Invasive Plant Sci Manag* 17:37–45
- Gerber E, Krebs C, Murrell C, Moretti M, Rocklin R, Schaffner U (2008) Exotic invasive knotweeds (*Fallopia* spp.) negatively affect native plant and invertebrate assemblages in European riparian habitats. *Biol Conserv* 141:646–654
- Germishuizen G (1986) Notes on African plants. *Bilderdykia* and *Reynoutria* new to the flora of the southern African region. *Bothalia* 16:233
- Goodwin S, McPherson JD, McCombie WR (2016) Coming of age: ten years of next-generation sequencing technologies. *Nat Rev Genet* 17:333–351
- Green PS (1966) Identification of the species and hybrids in the *Lonicera tatarica* complex. *J Arnold Arbor* 47:75–88
- Grimsby JL, Tsirelson D, Gammon MA, Kesseli R (2007) Genetic diversity and clonal vs. sexual reproduction in *Fallopia* spp. (Polygonaceae). *Am J Bot* 94:957–964
- Grob GB, Gravendeel B, Eurlings MCM (2004) Potential phylogenetic utility of the nuclear *FLORICAULA/LEAFY* second intron: comparison with three chloroplast DNA regions in *Amorphophallus* (Araceae). *Mol Phyl Evol* 30:13–23
- Hamming RW (1950) Error detecting and error correcting codes. *Bell Syst Technol J* 29:147–160
- Hastings WK (1970) Monte Carlo sampling methods using Markov chains and their applications. *Biometrika* 57:97–109
- Hollingsworth ML, Bailey JP (2000) Evidence for massive clonal growth in the invasive weed *Fallopia japonica* (Japanese Knotweed). *Bot J Linn Soc* 133:463–472
- Howarth DG, Baum DA (2005) Genealogical evidence of homoploid hybrid speciation in an adaptive radiation of *Scaevola* (Goodeniaceae) in the Hawaiian Islands. *Evolution* 59:948–961
- Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. *Mol Biol Evol* 23:254–267
- Iwatsubo Y, Kodate G, Naruhashi N (2004) Polyploidy of *Reynoutria japonica* var. *japonica* (Polygonaceae) in Japan. *J Phytogeogr Taxon* 52:137–142
- Johnson M, Zaretskaya I, Raytselis Y, Merezukh Y, McGinnis S, Madden TL (2008) NCBI BLAST: a better web interface. *Nucleic Acids Res* 36:W5–W9
- Jordon-Thaden IE, Spoelhof JP, Viccini LF, Combs J, Gomez Jr F, Walker I, Soltis DE, Soltis PS (2023) Phenotypic trait variation in the North American *Tragopogon* allopolyploid complex. *Am J Bot* 110:e16189
- Kadlečová M, Vojík M, Kutlvaš J, Berchová-Bímová K (2022) Time to kill the beast—Importance of taxa, concentration and timing during application of glyphosate to knotweeds. *Weed Res* 62:215–223
- Kartesz JT (2015) The Biota of North America Program (BONAP). <http://www.bonap.net/tdc>. Accessed: August 22, 2024
- Kim JY, Park CW (2000) Morphological and chromosomal variation in *Fallopia* section *Reynoutria* (Polygonaceae) in Korea. *Brittonia* 52:34–48
- Kim ST, Sultan SE, Donoghue MJ (2008) Allopolyploid speciation in *Persicaria* (Polygonaceae): insights from a low-copy nuclear region. *Proc Natl Acad Sci USA* 105:12370–12375
- Kuehne LM, Ostberg CO, Chase DM, Duda JJ, Olden JD (2020) Use of environmental DNA to detect the invasive aquatic plants *Myriophyllum spicatum* and *Egeria densa* in lakes. *Freshw Sci* 39:521–533
- Lavoie C (2017) The impact of invasive knotweed species (*Reynoutria* spp.) on the environment: review and research perspectives. *Biol Invasions* 19: 2319–2337
- Lawson JW, Fennell M, Smith MW, Bacon KL (2021) Regeneration and growth in crowns and rhizome fragments of Japanese knotweed (*Reynoutria japonica*) and desiccation as a potential control strategy. *PeerJ* 9:e11783
- Lecerf A, Patfield D, Boiché A, Riipinen MP, Chauvet E, Dobson M (2007) Stream ecosystems respond to riparian invasion by Japanese knotweed (*Fallopia japonica*). *Can J Fisheries Aquat Sci* 64:1273–1283
- Lubell JD, Brand MH, Lehrer JM, Holsinger KE (2008) Detecting the influence of ornamental *Berberis thunbergii* var. *atropurpurea* in invasive populations of *Berberis thunbergii* (Berberidaceae) using AFLP. *Am J Bot* 95:700–705
- Macas J, Kejnovský E, Neumann P, Novák P, Kobližková A, Vyskot B (2011) Next generation sequencing-based analysis of repetitive DNA in the model dioecious plant *Silene latifolia*. *PLoS ONE* 6:e27335
- Maddison WP, Maddison DR (2023) Mesquite: A Modular System for Evolutionary Analysis. Version 3.81. <https://www.mesquiteproject.org> Accessed: January 10, 2024
- Maddox V, Byrd J, Serviss B (2010) Identification and control of invasive privets (*Ligustrum* spp.) in the middle southern United States. *Invasive Plant Sci Manag* 3:482–488
- Maerz JC, Blossey B, Nuzzo V (2005) Green frogs show reduced foraging success in habitats invaded by Japanese knotweed. *Biodivers Conserv* 14:2901–2911
- Mandák B, Pyšek P, Bímová K (2004) History of the invasion and distribution of *Reynoutria* taxa in the Czech Republic: a hybrid spreading faster than its parents. *Preslia* 76:15–64
- Mandák B, Pyšek P, Lysák M, Suda JAN, Krahulcova A, Bímová K (2003) Variation in DNA-ploidy levels of *Reynoutria* taxa in the Czech Republic. *Ann Bot* 92:265–272
- Mclean S (2010) Identification of the presence and impact of Japanese knotweed on development sites. *J Build Apprais* 5:289–292
- Mendiburu F (2021) agricolae: Statistical Procedures for Agricultural Research. R Package Version 1.3.7. <https://CRAN.R-project.org/package=agricolae> Accessed: October 20, 2024
- Metropolis N, Rosenbluth AW, Rosenbluth MN, Teller AH, Teller E (1953) Equation of state calculations by fast computing machines. *J Chem Phys* 21:1087–1092
- Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, Lanfear R (2020) IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. *Mol Biol Evol* 37:1530–1534
- Mitchell N, Luu H, Owens GL, Rieseberg LH, Whitney KD (2022) Hybrid evolution repeats itself across environmental contexts in Texas sunflowers (*Helianthus*). *Evolution* 76:1512–1528
- Moody ML, Les DH (2002) Evidence of hybridity in invasive watermilfoil (*Myriophyllum*) populations. *Proc Natl Acad Sci USA* 99:14867–14871
- Moravcová I, Pyšek P, Jarošík V, Zákavský P (2011) Potential phytotoxic and shading effects of invasive *Fallopia* (Polygonaceae) taxa on the germination of native dominant species. *NeoBiota* 9:31–47
- Moyroud E, Tichtinsky G, Parcy F (2009) The *LEAFY* floral regulators in angiosperms: conserved proteins with diverse roles. *J Plant Biol* 52:177–185
- Müller K (2005) SeqState: primer design and sequence statistics for phylogenetic DNA datasets. *Appl Bioinf* 4:65–69
- Murrell C, Gerber E, Krebs C, Parepa M, Schaffner U, Bossdorf O (2011) Invasive knotweed affects native plants through allelopathy. *Am J Bot* 98: 38–43
- Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ (2015) IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol* 32:268–274
- Nishimoto Y, Ohnishi O, Hasegawa M (2003) Topological incongruence between nuclear and chloroplast DNA trees suggesting hybridization in the *urophyllum* group of the genus *Fagopyrum* (Polygonaceae). *Genes Genet Syst* 78:139–153
- Paradis E, Schliep K (2019) ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics* 35:526–528
- Parepa M, Fischer M, Krebs C, Bossdorf O (2014) Hybridization increases invasive knotweed success. *Evol Appl* 7:413–420
- Parepa M, Schaffner U, Bossdorf O (2012) Sources and modes of action of invasive knotweed allelopathy: the effects of leaf litter and tilled soil on the germination and growth of native plants. *NeoBiota* 13:15–30
- Park CW, Bhandari GS, Won H, Park JH, Park DS (2018) Polyploidy and introgression in invasive giant knotweed (*Fallopia sachalinensis*) during the colonization of remote volcanic islands. *Sci Rep* 8:16021



- Pearson K (1901) On lines and planes of closest fit to systems of points in space. *Lond Edinb Dubl Phil Mag* 2:559–572
- Randall JM, Morse LE, Benton N, Hiebert R, Lu S, Killeffer T (2008) The invasive species assessment protocol: a tool for creating regional and national lists of invasive nonnative plants that negatively impact biodiversity. *Invasive Plant Sci Manag* 1:36–49
- R Core Team (2024) R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing. <https://www.R-project.org>
- Rieseberg LH, Raymond O, Rosenthal DM, Lai Z, Livingstone K, Nakazato T, Durphy JL, Schwarzbach AE, Donovan LA, Lexer C (2003) Major ecological transitions in wild sunflowers facilitated by hybridization. *Science* 301:1211–1216
- Roulin A, Auer PL, Libault M, Schlueter J, Farmer A, May G, Stacey G, Doerge RW, Jackson SA (2013) The fate of duplicated genes in a polyploid plant genome. *Plant J* 73:143–153
- Saad L, Tiébré MS, Hardy OJ, Mahy G, Vanderhoeven S (2011) Patterns of hybridization and hybrid survival in the invasive alien *Fallopia* complex (Polygonaceae). *Plant Ecol Evol* 144:12–18
- Saltonstall K (2003) A rapid method for identifying the origin of North American *Phragmites* populations using RFLP analysis. *Wetlands* 23:1043–1047
- Sanchez A, Kron KA (2008) Phylogenetics of Polygonaceae with an emphasis on the evolution of Eriogonoideae. *Syst Bot* 33:87–96
- Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74:5463–5467
- Scheen AC, Pfeil BE, Petri A, Heidari N, Nylinder S, Oxelman B (2012) Use of allele-specific sequencing primers is an efficient alternative to PCR subcloning of low-copy nuclear genes. *Mol Ecol Res* 12:128–135
- Schierenbeck KA, Ellstrand NC (2009) Hybridization and the evolution of invasiveness in plants and other organisms. *Biol Invasions* 11:1093–1105
- Schuster TM, Wilson KL, Kron KA (2011) Phylogenetic relationships of *Muehlenbeckia*, *Fallopia*, and *Reynoutria* (Polygonaceae) investigated with chloroplast and nuclear sequence data. *Int J Plant Sci* 172:1053–1066
- Serniak LT, Corbin CE, Pitt AL, Rier ST (2017) Effects of Japanese knotweed on avian diversity and function in riparian habitats. *J Ornithol* 158:311–321
- Siemens TJ, Blossey B (2007) An evaluation of mechanisms preventing growth and survival of two native species in invasive Bohemian knotweed (*Fallopia × bohemica*, Polygonaceae). *Am J Bot* 94:776–783
- Simmons MP, Ochoterena H (2000) Gaps as characters in sequence-based phylogenetic analyses. *Syst Biol* 49:369–381
- Smith AL, Hodkinson TR, Villellas J, Catford JA, Csörgő AM, Blomberg SP, Crone EE, Ehrlén J, Garcia MB, Laine AL, Roach DA (2020) Global gene flow releases invasive plants from environmental constraints on genetic diversity. *Proc Natl Acad Sci USA* 117:4218–4227
- Suchard MA, Lemey P, Baele G, Ayres DL, Drummond AJ, Rambaut A (2018) Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evol* 4:vey016
- Tataridas A, Jabran K, Kanatas P, Oliveira RS, Freitas H, Travlos I (2022) Early detection, herbicide resistance screening, and integrated management of invasive plant species: a review. *Pest Manag Sci* 78:3957–3972
- Tavaré S (1986) Some probabilistic and statistical problems in the analysis of DNA sequences. *Lect Math Life Sci* 17:57–86
- Te Beest M, Le Roux JJ, Richardson DM, Brysting AK, Suda J, Kubešová M, Pyšek P (2012) The more the better? The role of polyploidy in facilitating plant invasions. *Ann Bot* 109:19–45
- Tippery NP, Harms NE, Purcell MF, Hong SH, Häfliger P, Killoy K, Wolfe AL, Thum RA (2023) Assessing the genetic diversity of *Nymphoides peltata* in the native and adventive range using microsatellite markers. *Biol Invasions* 25:3949–3963
- Tippery NP, Olson AL, Wendtlandt JL (2021) Using the nuclear *LEAFY* gene to reconstruct phylogenetic relationships among invasive knotweed (*Reynoutria*, Polygonaceae) populations. *Invasive Plant Sci Manag* 14:92–100
- Tippery NP, Pesch JD, Murphy BJ, Bautzmann RL (2020) Genetic diversity of native and introduced *Phragmites* (common reed) in Wisconsin. *Genetica* 148:165–172
- Tukey JW (1949) Comparing individual means in the analysis of variance. *Biometrics* 5:99–114
- Vrchotová N, Šerá B (2008) Allelopathic properties of knotweed rhizome extracts. *Plant Soil Environ* 54:301–303
- Weigel D, Alvarez J, Smyth DR, Yanofsky MF, Meyerowitz EM (1992) *LEAFY* controls floral meristem identity in *Arabidopsis*. *Cell* 69:843–859
- Welles SR, Ellstrand NC (2020) Evolution of increased vigour associated with allopolyploidization in the newly formed invasive species *Salsola ryanii*. *AoB Plants* 12:plz039
- Wendell DL, Huang X, Gryspeerd B, Freeland J (2021) A simple screen to detect hybrids between native and introduced *Phragmites australis* in the United States and Canada. *J Great Lakes Res* 47:1453–1457
- Wickham H (2016) *ggplot2: Elegant Graphics for Data Analysis*. New York: Springer-Verlag. 213 p
- Yan P, Pang QH, Jiao XW, Zhao A, Shen YJ, Zhao SJ (2008) Genetic variation and identification of cultivated *Fallopia multiflora* and its wild relatives by using chloroplast *matK* and 18S rRNA gene sequences. *Planta Med* 74:1504–1509
- Yang S, Ferrari MJ, Shea K (2011) Pollinator behavior mediates negative interactions between two congeneric invasive plant species. *Am Nat* 177:110–118
- Yoshimoto A, Szűcs M (2024) Could hybridization increase the establishment success of the biological control agent *Aphalara itadori* (Hemiptera: Aphalaridae) against invasive knotweeds? *Ecol Evol* 14:e10936
- Zika PF, Jacobson AL (2003) An overlooked hybrid Japanese knotweed (*Polygonum cuspidatum* × *sachalinense*; Polygonaceae) in North America. *Rhodora* 105:143–152