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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 (*R. japonica* Houtt., *R. sachalinensis* (F.Schmidt) Nakai) and their hybrid (known as *R.* × *bohemica* Chrtek & Chrtková) 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 there is not only one kind of interspecific hybrid, but in fact 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 knotweeds 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.

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

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Management Implications

Invasive knotweeds pose a threat to ecosystems across Europe and North America. There are numerous genetic entities in the invaded range, including two species (Japanese knotweed and giant knotweed) and several distinct kinds of hybrids (collectively known as Bohemian knotweed). 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 identity of their invasive knotweed 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 knotweeds. 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 knotweed plants.

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. (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 (*R. japonica* Houtt.), giant knotweed (*R. sachalinensis* (F.Schmidt) Nakai), and their hybrid known as bohemian knotweed (*R.* × *bohemica* Chrtek & Chrtková) have similar invasive potential in temperate habitats, owing to their aggressive clonal growth (Bailey

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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 knotweed species include ecosystem disruption (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 of 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.* × *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 knotweeds 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 knotweed 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 knotweeds, 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

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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 over 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 *R. compacta* (Hook.f.) Nakai and *R. multiflora* (Thunb.) Moldenke (Desjardins et al. 2023a; Park et al. 2018; Tippery et al. 2021). Analysis of *LEAFY* second intron sequences for invasive knotweeds in the USA 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.* × *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 of 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 data set 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 (Fig. 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

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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.

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, 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 Tippery et al. (2020). PCR was conducted using the Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.), with a 58ºC 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 LLC (Louisville, Kentucky, U.S.A.). In situations where Sanger sequencing produced polymorphic results, separate sequences were obtained either by subcloning PCR 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 Corporation, Madison, Wisconsin, U.S.A.) and cloned into bacteria using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific), then colonies of transformed cells were subjected to

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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 LLC 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; Tippery et al. 2021) and aligned manually using Mesquite ver. 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). Sequence alignments were analyzed using the *ape* package ver. 5.7.1 (Paradis and Schliep 2009) for R ver. 4.4.1 (R Core Team 2024). After model selection using IQ-TREE ver. 2.0.5 (Minh et al. 2020; Nguyen et al. 2015), phylogenetic analyses were conducted in BEAST ver. 1.10.4 (Suchard et al. 2018) with the GTR+G model of evolution (Tavaré 1986), using ten 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.

In order 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 ver. 1.4.1 (Müller 2005). After removing identical sequences from the data matrix, genetic distances among *LEAFY* gene sequences were visualized using the program SplitsTree ver. 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 together 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).

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Diagnostic PCR

Primers for diagnostic PCR reactions were developed by identifying regions that consistently differed among types of *Reynoutria* sequence. 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 above for other primers, and amplicons were run using electrophoresis on a 2% agarose gel containing GelStar™ nucleic acid stain (Lonza Bioscience, Walkersville, Maryland, USA). PCR products (4 µL of each) were run alongside 2 µL of GeneRuler 1 kb Plus DNA ladder (Thermo Fisher Scientific).

Morphology analyses

Morphological data were evaluated for individuals with known *LEAFY* gene sequences. Data were visualized using the *ggplot2* package ver. 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 analysis of variance (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 ver. 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 (Fig. 1; Supplementary Table S1). Five unique sequence variants were identified, and these were assigned codes that corresponded to previously identified variants (Tippery 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 variants obtained for a single individual as its 'composite

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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–S2).

Sequence alignment for the *LEAFY* second intron was 1,149 nucleotides in length and included 26 polymorphic sites and 42 indels (Fig. 2). The first intron was 483–528 nucleotides in length and included 19 polymorphic sites and 10 indels. The second exon was 402–408 nucleotides in length and included five polymorphic sites and one indel. The second intron was 599–1056 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 (Fig. 2). Sequence data for the first exon were incomplete but comprised 240 nucleotides, of which three sites were polymorphic, with no indels. Because of the location of the MLFYI2-2385R 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 (Figs. 3; Supplementary Figures S1–S2). Newly reported sequence variants frequently were most similar to previously reported sequences from invasive plants in New Zealand or the UK. 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 UK. Notably, the J1 variant did not share close similarity with any previously published sequences from outside the USA. The phylogenetic position of *R. compacta* relative to *R. japonica* and *R. sachalinensis* was poorly supported and inconclusive.

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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* Ohsako & Ohnishi (GenBank accession number 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* (Fig. 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 two *R. japonica* sequence variants was 30 differences, and the maximum distance between *R. sachalinensis* sequence variants was five differences.

Diagnostic PCR reactions corroborated the genotype evidence that was obtained using DNA sequencing (Fig. 5). The expected lengths of PCR products were as follows: Japo1F/MLFYI2-2385R: 638–678 bp, Sach1F/MLFYI2-2385R: 548–549 bp, Reyn3F/Japo1R: 1102–1106 bp, Reyn3F/Sach1R: 1055–1091 bp, Individuals that had only *R. japonica* sequence variants failed to amplify products using the Sach1F or Sach1R primers, individuals with only *R.*

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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 (Fig. 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 been seldom sequenced in angiosperms. We were able to obtain sequence data from these regions for all previously identified genetic variants in the USA, 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 (Fig. 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 length data was 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 8–29 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 (Tippery 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 one or the other parental species (Jordon-Thaden et al. 2023; Mitchell et al. 2022;

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Rieseberg et al. 2003). Thus, it is important to evaluate morphological differences among knotweed 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 (Fig. 6). Long abaxial 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 (Fig. 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 (Fig. 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 (Fig. 6B). Notably, this was the only hybrid composite genotype with an S2 sequence variant. Male-sterile plants were rarely encountered among hybrids (Fig. 6E). Lamina length was widely variable overall (Fig. 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: $F(7,143) = 22.37$, $p < 2^{-16}$). The J1/S1 and J2/S2 hybrids had a significantly different mean for their lamina base morphology (Fig. 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 morphology 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 analysis (Fig. 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

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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* (Figs. 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 (Bímová et al. 2003; Mandák et al. 2004; Moravcová et al. 2011). Our study corroborates prior evidence that the invasive USA knotweed 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.* × *bohemica* (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.* × *bohemica*" may hinder a more nuanced understanding of hybridization in *Reynoutria*, and we support efforts to distinguish hybrids by their genetic composition rather than simply their species parentage (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 UK (Figs. 3–4). 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

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sequence from outside the USA, and this could indicate that the variant is only present in USA 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 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 non-functional, 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 to *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 from both methods. 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 more rapid 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

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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 knotweeds 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.

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Data availability

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

Competing Interests

Competing interests: The authors declare none.

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Table 1. DNA sequences for oligonucleotide primers that were used to amplify portions of the *LEAFY* gene in *Reynoutria* species. Intron 1 is located at alignment positions 299–834, and intron 2 is located at positions 1223–2375.

Name	**Sequence**	**Location**	**Alignment position**	**Source**
FLint1-F1	5’-RGAGTTATTCCARGCTTACGG-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’-GGGAGAGAAAGAAGTGGTCCG-3’	Exon 2	849	This study
Japo5R	5’-GTGCAACTTCTCCAGGCTCC-3’	Exon 2	1110	This study
Sach1F	5’-AGCTAGTTAGCTAGGTAGCTAC-3’	Intron 2	1325	This study
Sach1R	5’-TTCATATTTGTAGCTACCTAGC-3’	Intron 2	1334	This study
Japo1F	5’-GGTAAAGTGAGAGACGTATAAATG-3’	Intron 2	1338	This study
Japo1R	5’-CATTTATACGTCTCTCACTTTACC-3’	Intron 2	1338	This study
MLFYI2-2385R	5’-TGCGTAYCTGAACACTTGGTTYGT-3’	Exon 3	2379	Schuster et al. 2011

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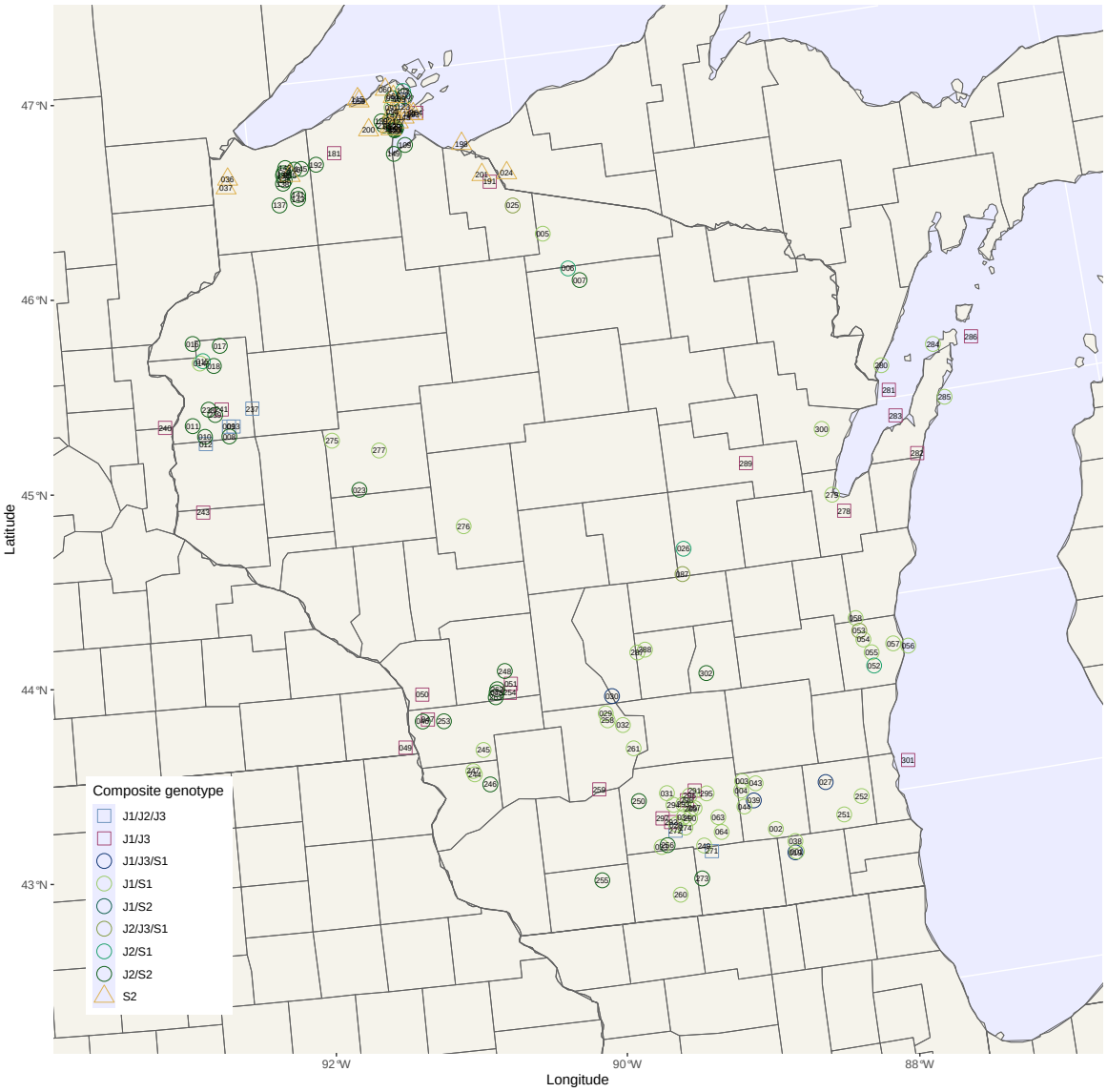

Figure 1. Map of *Reynoutria* collection sites in Wisconsin. Shapes indicate taxonomic identity (square = *R. japonica*, circle = *R.* × *bohemica*, triangle = *R. sachalinensis*), and colors depict unique composite genotypes. Numbers inside the shapes refer to specimen ID (Supplementary Table S1). Site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

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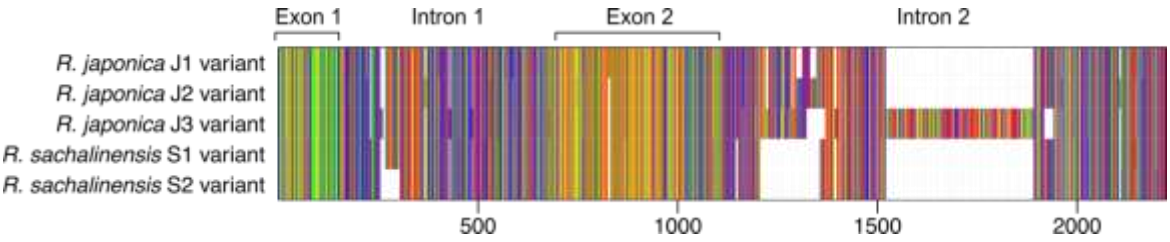

Figure 2. Alignment of *LEAFY* sequences for invasive *Reynoutria* taxa, showing the most commonly encountered sequences for each sequence variant. Sequences are identified as originating from *R. japonica* (J1 / J2 / J3) or *R. sachalinensis* (S1 / S2). Intron and exon borders are indicated; the third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

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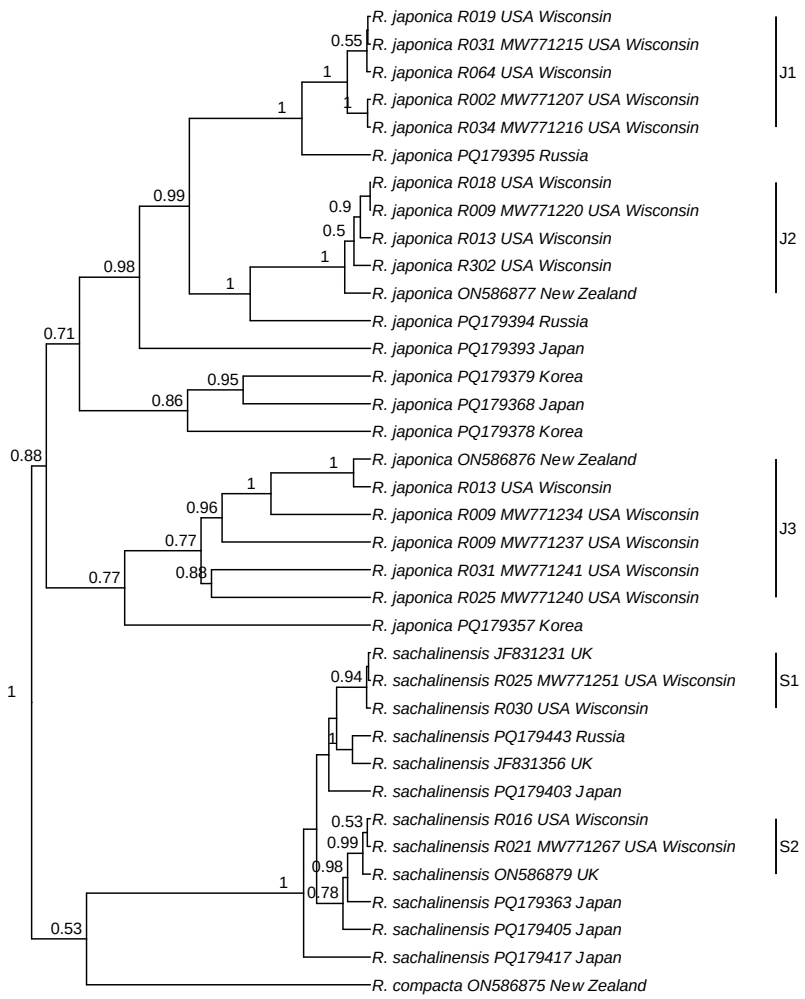

Figure 3. Phylogenetic relationships among sequences obtained from *Reynoutria* species. Sequences are identified to species according to their previous identifications (Schuster et al. 2011; Park et al. 2018; Desjardins et al. 2023a) or by their similarity to previously published sequences. Previously published sequences are labeled with GenBank accession numbers. Sequences from plants in Wisconsin (this study; Tippery et al. 2021) have a unique identifier (e.g., 'R019') that is referenced in the supplementary appendix (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.

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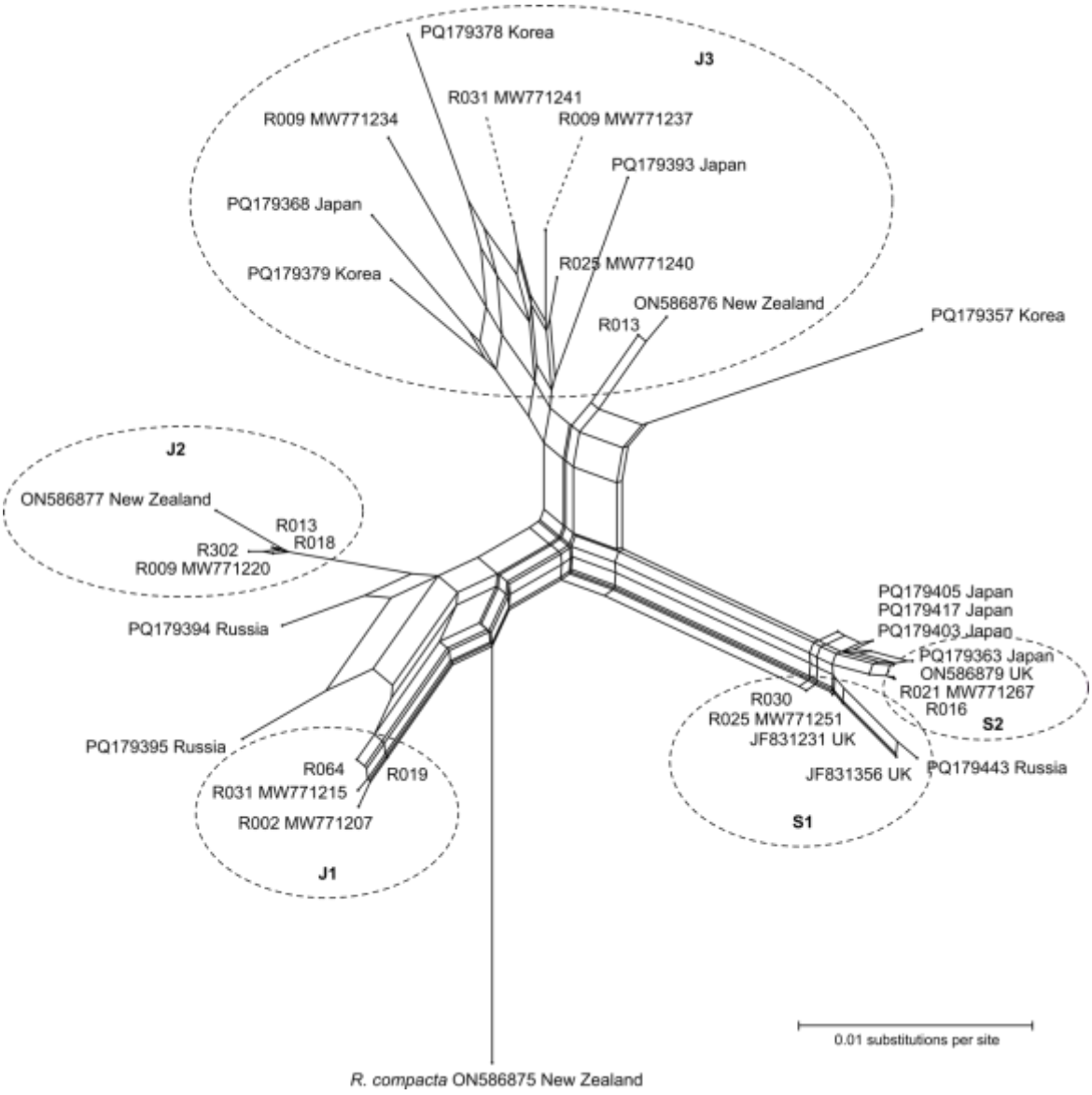

Figure 4. SplitsTree network of *LEAFY* sequences. Each label represents a unique sequence variant that is connected to similar sequences by lines. Previously published sequences are identified with GenBank accession numbers. Sequences from plants in Wisconsin (this study; Tippery et al. 2021) have a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

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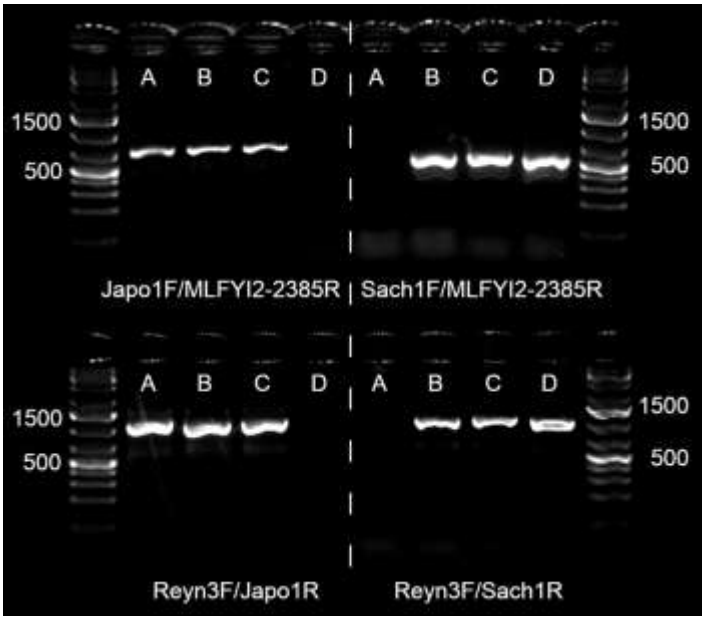

Figure 5. Results of diagnostic PCR reactions using primers that selectively amplify either *R. japonica* or *R. sachalinensis* sequence variants. The top row shows the result of two primer pairs, separated by a dotted line, both using the MLFYI2-2385R reverse primer located in the *LEAFY* third exon, 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.

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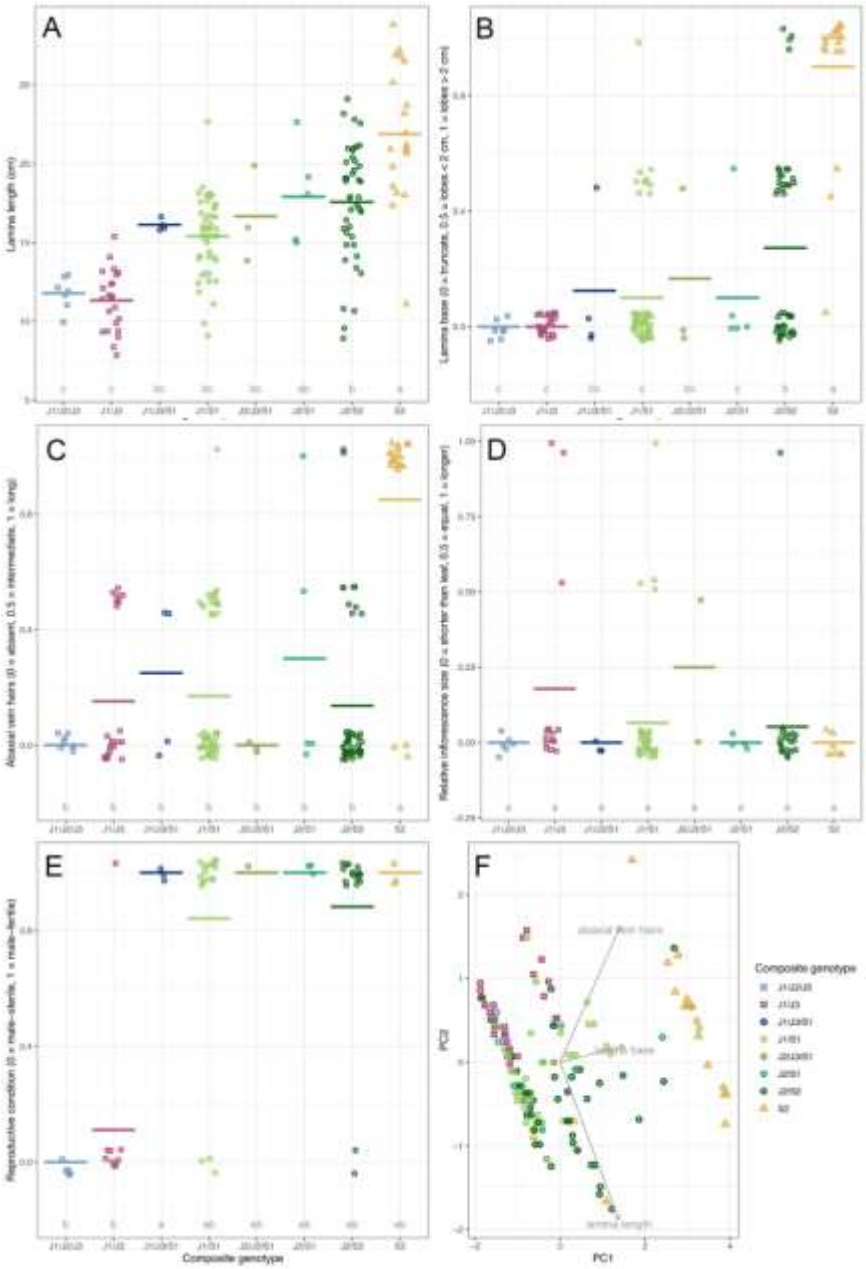

Figure 6. Morphological data for plants with known composite genotypes. In each panel, shapes correspond to taxonomic identity (square = *R. japonica*, circle = *R.* × *bohemica*, triangle = *R. sachalinensis*), and colors depict unique composite genotypes. Positions are 'jittered' by a value of 0.2 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, (E) reproductive condition. The final panel (F) shows the principal component analysis, with vectors showing the relative contributions of the three morphological traits that were evaluated.

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