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Research Article

A knot of hybrids: Differentiating Asian knotweeds in North-Eastern France using genetic, cytological, and morphological data

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Abstract The two invasive *Reynoutria* species, *Reynoutria japonica* var. *japonica* and *Reynoutria sachalinensis*, and their hybrid *Reynoutria x bohémica* are often misidentified by managers and nonspecialists. The taxonomic confusions are all the more exacerbated by the infraspecific variability of introduced populations in terms of morphology, genetic diversity, and ploidy level. We resolved the identity of North-Eastern French invasive populations using 4582 single-nucleotide polymorphisms (SNPs) from a RADseq analysis, DNA contents estimated by flow cytometry, and 12 vegetative morphometric variables. The SNPs supported only one single genotype for *R. japonica* over 11 localities, while the nine localities of *Reynoutria x bohémica* were represented by one genotype each. Estimation of genome size using DAPI staining and flow cytometry revealed only octoploid cytotypes for *R. japonica* and hexaploid cytotypes for *R. x bohémica*, whereas *R. sachalinensis* was represented by tetraploid and hexaploid cytotypes. Among morphometric variables, no single one allows for a clear differentiation of the three taxa. We propose a combination of characters to easily and quickly identify these three invasive taxa based on six vegetative criteria including leaf and apex length, as well as leaf shape, leaf base, and apex shape, and the extrafloral nectaries on the node.

Key words: *Fallopia* spp, flow cytometry, invasive plant species, morphometric analysis, population genetics, RADseq, *Reynoutria* spp.

1 Introduction

The genus *Reynoutria* Houtt. (Polygonaceae), sometimes referred to as *Fallopia* Adans., comprises five Eastern Asian species, with three of them showing invasiveness in various temperate regions worldwide: *Reynoutria japonica* var. *japonica* Houtt. (hereafter named *R.jap*), *Reynoutria sachalinensis* (F.Schmidt) Nakai (*R.sac*), and their hybrids named *Reynoutria x bohémica* Chrtek & Chrtková (*R.x.boh*). In their introduced area, these geophytic rhizomatous perennial herbs mainly reproduce and disperse clonally by rhizomes and stem fragmentation (Aguilera et al., 2010). They complete their vegetative cycle between March and late November, in North-Eastern (NE) France. Flowering occurs in August, followed by a fructification period until the end of the cycle, ultimately accumulating reserves in the rhizome year after year (Jones et al., 2018). Sexual reproduction has also been observed, mediated by generalist pollinators (Johnson et al., 2019), contributing to their expansion (Bímová et al., 2003). Knowing that knotweeds are gynodioecious species, *R.jap* is represented by a single octoploid female clone in Europe, preventing intraspecific sexual reproduction (Hollingsworth & Bailey, 2000; Mandák et al., 2005). However,

R.sac and *R.x.boh* exist in both female and hermaphroditic forms (Tiébré et al., 2007a), thus permitting multiple pollination events. *R.jap* can cross-pollinate with hermaphroditic forms of *R.sac* and *R.x.boh*, both of which produce viable pollen; similarly, there is evidence of backcrossing between *R.sac* and *R.x.boh*, as well as cross-fertilization between hybrid *R.x.boh* (Tiébré et al., 2007b). Indeed, a study conducted in the Czech Republic identified *R.x.boh* as the most genetically diverse taxon among the three (Mandák et al., 2005). In NE France, *R.x.boh* samples were described as hexaploid and more rarely octoploid with additional aneuploid variation (Mandák et al., 2003; Schnitzler & Bailey, 2008). *R.sac* is predominantly tetraploid (Mandák et al., 2003; Suda et al., 2010).

In Europe, these invasive taxa create various ecological and economic challenges, especially in riparian areas along waterways (Sarat et al., 2015; Collier et al., 2020). Besides, they are considered as allelopathic, granting them a competitive edge over native species in disturbed ecosystems (Kato-Noguchi, 2022). Areas invaded by Asian knotweeds experience decreased biodiversity and undergo alterations in plant communities due to the inhibition of root growth and germination of native plants (Aguilera et al., 2010;

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Šoln et al., 2022). The allelopathic effects of Asian knotweed extend to ecosystem services, affecting soil food webs, mycorrhizal relationships, and nutrient availability (Abgrall et al., 2018; Pinzone et al., 2018; Zubek et al., 2022). Asian knotweeds also inflict economic damages to human activities such as agriculture (Dommanget et al., 2019). Thus, these taxa represent a significant damage cost but also a strong management cost. For example, Asian knotweeds have been calculated to cost £165 609 000 per year in the United Kingdom (Diagne et al., 2021; Hocking et al., 2023). Consequently, the management and control of these invasive taxa pose significant pressing challenges for terrestrial and aquatic ecosystems in Europe.

To effectively lead management and control operations, it is crucial to distinguish among these three taxa clearly. For this, some criteria are well-known and particularly discriminant like leaf indumentum (Mered'a et al., 2019), the abaxial epidermal trichomes (Pashley, 2003; Bailey et al., 2009), inflorescence structures (Bailey & Wisskirchen, 2004), and epidermal ornamentation (Desjardins et al., 2023). However, these criteria are more difficult to comprehend and may require specific equipment, knowledge, or period. Besides, *R.x.boh* is known for its high phenotypic variability and intermediate morphology resulting from its hybrid nature between the two parental species (Bímová et al., 2004; Dommanget et al., 2019). This variability is equally enhanced by adaptation to concurrent native species for resource availability (Davidson et al., 2011). Consequently, numerous identification errors were found for this species complex, especially between *R.jap* and *R.x.boh*, in regional or international websites that support biodiversity data use such as the Natural Heritage Inventory Information System (SINP) or the Global Biodiversity Information Facility (GBIF, 2022). The NE France is particularly affected by Asian knotweed invasion, and hybridization produces a clinal rather than a binary morphological variation (Schnitzler & Bailey, 2008).

Thus, we aimed to propose some simple morphological vegetative criteria like extrafloral nectaries, stem coloration, leaf shape, and leaf length, which are already widely applied (Bailey & Wisskirchen, 2004; Mered'a et al., 2019). All these criteria permit well-identified *R.sac* but may be less effective to distinguish *R.x.boh* from its parent species *R.jap* (Bailey et al., 2009; Mered'a et al., 2019). The primary objective of this study is to differentiate the three taxa, with a particular focus on distinguishing *R.x.boh* from *R.jap*. In NE France using several taxonomic markers in an integrative approach: (i) determining the genetic and taxonomic identity of different populations through the utilization of highly sensitive molecular markers, such as single-nucleotide polymorphisms (SNPs) identified through RADseq analysis, (ii) testing the resulting genetic clusters against simple and vegetative morphological criteria to identify the most reliable ones for distinguishing the three taxa studied, and (iii) gaining a deeper insight into the genetic clustering by estimating ploidy variation in sampled populations through the application of flow cytometry.

2 Material and Methods

2.1 Plant material and preliminary identification

Plant material was collected through 26 populations located in NE France (Region Alsace), France (Table S1). Five of them were identified as *R.sac* through morphological field

identification following the criteria outlined by Bailey & Wisskirchen (2004). This identification relied solely on vegetative characters observed during the sampling period, including spots on the shoots, leaf base, leaf apex, extrafloral nectaries, leaf form, main stem size, height, and leaf structure. For each population, we harvested two transplanted specimens, consisting of rhizomes and aerial parts. These specimens were subsequently cultivated in 8 L pots filled with half of clay soil and half of highly organic potting soils, at the Botanical Garden of the University of Strasbourg (48.5832°N, 7.768082°E). Also, some samples of the populations studied are deposited in a public herbarium. These samples are identified with a barcode and are referenced in the herbarium of the University of Strasbourg.

The 21 remaining populations were definitively not associated with *R.sac* based on the mentioned characters; consequently, this group comprised either *R.jap* or *R.x.boh*. To assign a specific taxon within this group, RADseq analysis was used, with population R22 serving as the genotype control for *R.sac* to genetically differentiate the hybrid *R.x.boh* from *R.jap*. After RADseq analysis, we randomly identified 11 genotypes corresponding to *R.jap* and nine to *R.x.boh*. Subsequently, we decided to test multiple morphometric criteria for these results.

2.2 DNA extraction

Genomic DNA extraction was performed following the procedure of Doyle & Doyle (1987), with the following modifications. For each population, 30 mg of intact dried ground leaf tissue was incubated 1 h at 65 °C in 1 mL of extraction buffer (14 mL of NaCl, 5 mL of tris hydrochloride (Tris HCl), 2 g of cetyltrimethylammonium bromide (CTAB powder), and 29 mL of H₂O. 0.7 mL of chloroform:isoamyl alcohol (24:1) was then added. After 30 s of vortex mixing, samples were centrifuged for 10 min at 13 000 rpm at 4 °C. The aqueous phase was transferred and mixed with 0.6 mL of NaOAc–ethanol solution (3:12). After DNA precipitation at –20 °C overnight, DNA pellets were centrifuged and washed with Ethanol two times, and resuspended in Tris-EDTA with RNase.

2.3 RADseq and bioinformatic analysis

The *SimRAD* R-package (Lepais & Weir, 2014) was used to estimate the total number of reads necessary to achieve a mean sequencing depth of 12 reads per locus per sample. Microsynth Ecogenics (Balgach, Switzerland) generated RAD libraries using the EcoRI and MseI enzyme combination following Peterson et al. (2012), including the use of sample-specific barcodes. Samples were sequenced on an Illumina NextSeq platform to produce 150-bp single-end reads based on the EcoRI restriction site. The Illumina sequencing produced about 2–4 million reads per sample. The program *ipyrad* v.0.9.83 (Eaton & Overcast, 2020) was used to demultiplex raw reads, removing adapter sequences, trimming bases with Phred scores <31, and removing reads shorter than 50 bp after trimming. Some samples were removed based on their limited number of reads. Trimmed reads were assembled de novo using a cluster threshold of 95, a minimum of four samples per locus, and a maximum number of two alleles per locus for consensus reads. These analyses were conducted using the high-performance computing center of the University of Strasbourg.

Phylogenetic relationships were estimated in a UPGMA (unweighted pair group method with arithmetic mean) tree using the `bitwise.dist()` R-function from *adegenet* R-package (Jombart & Ahmed, 2011) in R environment (R Core Team, 2022). Samples were clustered in different multilocus genotypes to determine genetic clones using as a clonal threshold the maximal genetic distance between one sample and its technical replicates among four couples of sample–replicate, with the `mlg.filter()` R-function from *poppr* R-package (Kamvar et al., 2014) (Fig. S1). Genetic structure and admixture were inferred based on individual ancestry coefficients, and the choice of the most suitable number of ancestral populations (K) was based on the cross-entropy criterion, `snmf()` R-function in *LEA* R-package (Frichot & François, 2015).

2.4 Flow cytometry and ploidy levels

Flow cytometry was used to determine DNA contents and estimated ploidy levels using DAPI (4',6-diamidino-2-phenylindole) fluorochrome staining (Suda et al., 2010). For two independent biological replicates in each population, 1 cm² of young healthy leaves was collected closest to the apex. After collection, the samples were immediately immersed in liquid nitrogen and then preserved at -80°C . Nuclear DNA content was measured using the Sysmex CyStain UV[®] Precise P Kit (Ref: 05-5002, Sysmex Partec GmbH; Gôrlitz, Germany). Nuclei were released in nuclei extraction buffer by lightly chopping the material with a razor blade, stained with DAPI, and filtered through a CellTrics[™] 30 μm mesh, adapted for *Reynoutria* spp. (Mandák et al., 2003). About 30 000 isolated nuclei were used for each sample using the Attune[™] Flow Cytometer and Attune[™] Cytometer software (Life Technologies Corporation, Carlsbad, CA, USA) recording the relative fluorescence intensities. The DNA content was estimated for two replicates per population as a relative genome size (RGS) in arbitrary units. According to the literature, three cytotypes are mainly found for the European Asian knotweeds, that is, tetraploid ($2n=44$), hexaploid ($2n=66$), and octoploid ($2n=88$) (Mandák et al., 2003; Suda et al., 2010; Mered'a et al., 2023). A hierarchical ascendant classification was performed on the RGS variable using the *FactoMineR* R-package (Lê et al., 2008) with $K=3$ clusters, corresponding to the three cytotypes predominantly identified in Europe.

Subsequently, the samples were allocated to four clusters according to their RGS and their taxonomy.

2.5 Morphometric analysis

The morphological differentiation among *Reynoutria* taxa was evaluated using 12 morphological characters targeting leaves, stem, nodes, internodes, and extrafloral nectaries (Figs. 1, S2). Additionally, two ratio-based characters were computed in conjunction with the others: (i) the leaf shape (LSH) corresponding to the ratio between leaf apex length (LAL) and leaf blade length (LBL) (from an ovate to elliptic-oblong blade) and (ii) the apex shape (ASH) as the ratio between the maximum leaf width (MLW) and LBL (from an acute to cuspidate apex). The criterion, trichomes type, often presented as a discriminatory criterion (Pashley, 2003; Bailey & Wisskirchen, 2004), was not considered due to the requirement of specific equipment.

As far as possible, three ramets were examined at each locality. For each ramet, one stem, two internodes, three nodes, and three leaves were evaluated, selecting the largest and most developed leaves (Kim & Park, 2000; Bailey et al., 2009) in the central part of the stem (Zika & Jacobson, 2003; Desjardins et al., 2023). Also, due to the weak specification of inflorescence-associated leaves in *Reynoutria* genus (Bailey et al., 2009), these were not considered for the morphological analysis. Morphometric variation was analyzed with boxplots, adjusted R^2 , and discriminant analysis based on a principal component analysis using `discrim()` and `dudi.pca()` R-functions from *ade4* R-package (Bougeard & Dray, 2018).

3 Results

3.1 RADseq analysis and genetic admixture

The defined ipyrad pipeline parameters generated 588 853 variants, reduced to 4582 SNPs after reducing the amount of missing data to 10%. Admixture structure analysis distinguished two ancestral populations based on the cross-entropy criterion (Figs. 2B, 2C). The admixture analysis and the UPGMA tree described three distinct groups (Figs. 2A, 2B). Two of them essentially represented the ancestral populations. The first group included only a single sample initially attributed to *R.sac* in our sampling, while the second group comprised 11 samples, all of which logically aligned

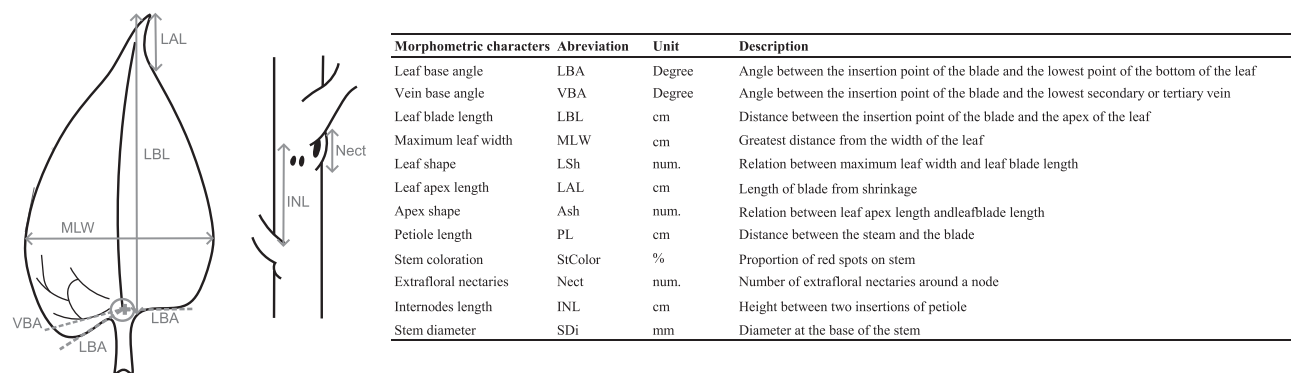


Fig. 1. Description of morphometric variables used to distinguish the three taxa of *Reynoutria* spp. based on vegetative parts.

with *R.jap*. The third cluster comprised nine samples, each representing a distinct genotype characterized by admixed compositions from the two ancestral populations. Within this cluster, there was a clear predominance of alleles from *R.sac*. These nine admixed genotypes were indicative of the hybrid taxon *R.x.boh*. Also, the distribution of genetic distances, based on the maximal distances between two replicates, was analyzed and confirmed the occurrence of different multi-locus genotype delimitations.

3.2 Genome size and ploidy levels

Considering the existence of three cytotypes in the literature, the hierarchical ascendant classification, with a cutoff value of three (for three possibilities of cytotypes), on the RGS placed every sample of *R.jap* in the same cluster. Populations R22 and R26 of *R.sac* form a second cluster. Populations R23, R24, and R25 of *R.sac* form a third cluster with all *R.x.boh* samples. Consequently, two cytotype groups for *R.sac* are proposed in Table 1. With the two groups of *R.sac*, we proposed four clusters in total for all our populations.

Assigning cytotypes to clusters aligns with the literature. *R.jap*, having the highest RGS, is assigned octoploid. *R.x.boh*, predominantly hexaploid, is assigned hexaploid, with a higher standard deviation (Table 1). For *R.sac*, the clustering divides the samples between two cytotypes. The clusters with values similar to *R.x.boh* are designated hexaploid and correspond to R23, R24, and R25. The cluster of *R.sac* with the lowest RGS is assigned tetraploid, consistent with the literature, and corresponds to R22 and R26.

3.3 Morphological differentiation

Despite a clear tridirectional structuring of the three taxa, discernible overlaps were also present on the first plan of the discriminant analysis (Fig. 3A). The first axis separated *R.jap* from *R.sac* and this axis was correlated with the stem coloration (St.Color) and the LSh. Also, this first axis was anticorrelated with the leaf base angle (LBA), the vein base angle (VBA), the LBL, the LAL, and the Ash. On the first axis, *R.x.boh* was considered intermediate compared to the other two species. The second axis separated *R.x.boh* from *R.jap* and *R.sac*. This second axis was correlated with the extrafloral nectaries number (Nect).

Among the 12 morphological characters tested, none of them could strictly distinguish the three taxa on their own,

none of the R^2 values approached the value one ($R^2 < 0.5$), and none of the taxa showed a boxplot that did not overlap with another (Fig. 3B). Besides, some traits with a low R^2 (less than 0.1) were not relevant. The variances of MLW, petiole length, stem diameter, and internode length were not discriminant traits ($R^2 < 0.07$ for all three taxa, and $R^2 < 0.08$ for *R.jap* and *R.x.boh*) (Fig. 3B). These morphological criteria appeared to be more conservative among the three taxa and did not permit the differentiation of any studied taxa.

Nevertheless, some variables allowed us to differentiate one taxon from the other two. The leaf base angle (LBA) showed that *R.sac* and *R.x.boh* presented mainly cordate leaves and *R.jap* a majority of truncate leaves; *R.x.boh* also shows a moderate number of truncate leaves. The vein base angle (VBA) showed the same pattern as LBA. In terms of the LBL, *R.sac* had the longest leaves. The LSh showed a higher ratio with *R.x.boh* and *R.jap*, so in these two taxa, the majority of leaves were ovate. For *R.sac*, the leaves were mostly elongated and elliptic. The LAL and the Ash concerning *R.sac* indicated a more pronounced and acute apex compared to *R.jap* and *R.x.boh*. The *R.jap* stem coloration (St.Color) was mainly reddish or densely red-brown spotted. In contrast, *R.sac* stems were not, or sporadically, red-brown spotted. *R.x.boh* showed an intermediate pattern. The

Table 1 Relative genome size (in arbitrary units; mean \pm SD) and estimated ploidy levels in *Reynoutria* spp. from 26 populations.

Taxon	Tetraploid (4x)	Hexaploid (6x)	Octoploid (8x)
<i>R.jap</i>	-	-	30.05 \pm 3.17 (n = 11)
<i>R.x.boh</i>	-	17.78 \pm 6.15 (n = 9)	-
<i>R.sac</i>	6.65 \pm 0.07 (n = 2)	16.71 \pm 1.91 (n = 3)	-

Data are based on the DNA content of the G0/G1 nuclei, determined by flow cytometry after DAPI staining. The numbers of populations analyzed are shown in parentheses. Taxon column based on RADseq analysis (except for R23, R24, R25, and R26 identification according to Bailey & Wisskirchen, 2004).

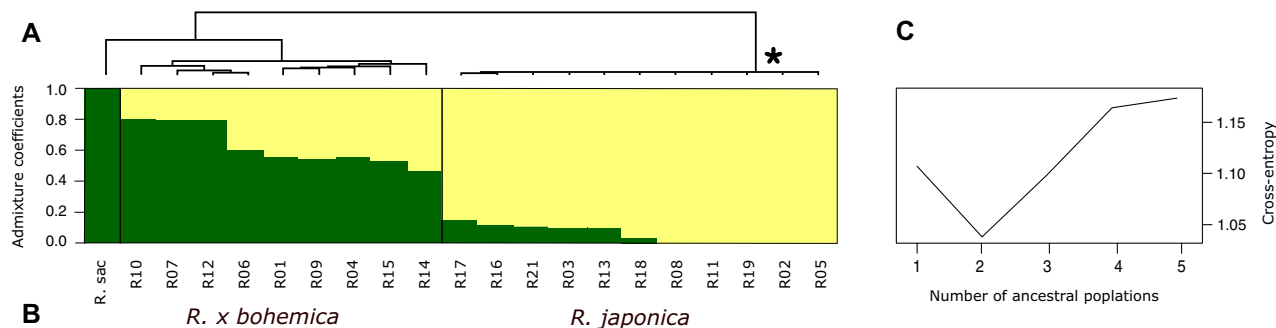


Fig. 2. Genetic structure and admixture of the *Reynoutria* taxa based on 4582 single-nucleotide polymorphisms obtained by a RADseq analysis. **A**, UPGMA tree. *, only one multilocus genotype for all *R.jap* samples. **B**, admixture analysis. **C**, optimal number of ancestral populations ($K = 2$) according to the cross-entropy criterion. *R.sac* corresponds to R22 (tetraploid).

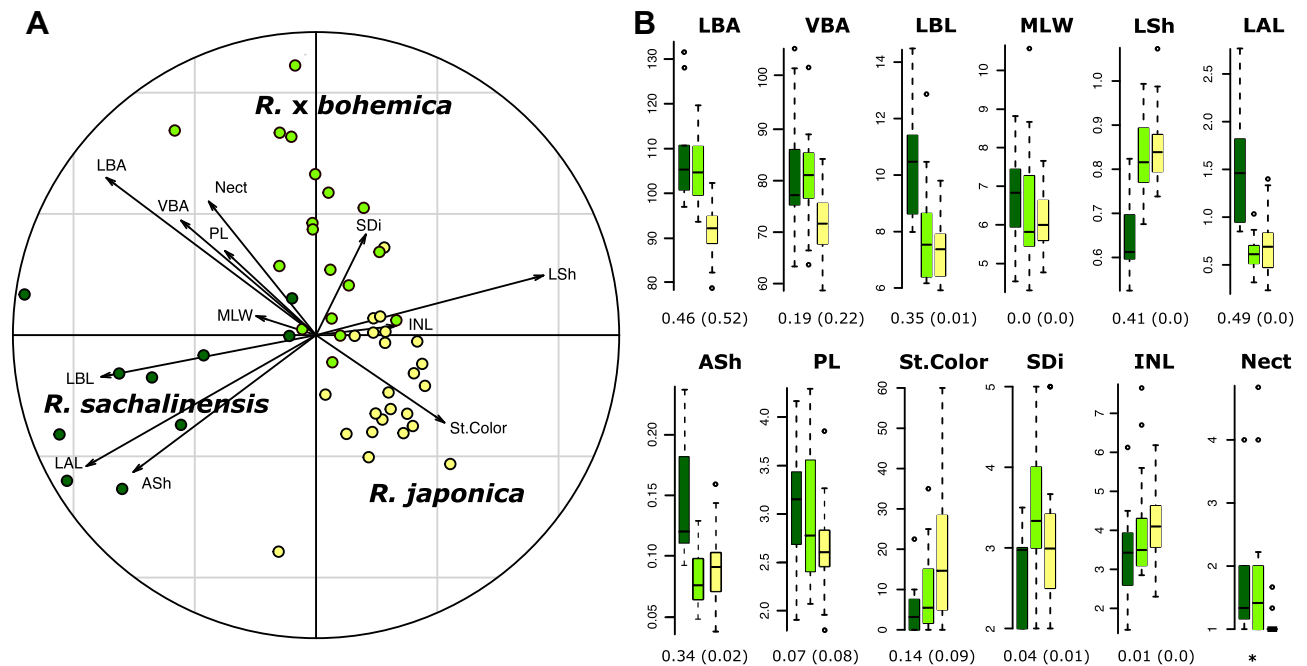


Fig. 3. Morphological analysis based on the mean values of 26 populations of *Reynoutria* spp. for 12 morphometric variables. **A**, Discriminant analysis. **B**, Boxplots of the variability of morphological characters between the three taxa, with adjusted R^2 estimated between the three taxa (in brackets, between *R.jap* and *R.x.boh*). Yellow circles and boxplots, *R.jap* ($n = 22$); Dark green circles and boxplots, *R.sac* ($n = 10$); green circles and boxplots, *R.x.boh* ($n = 18$). *, discrete variable, R^2 not estimated. Abbreviations of the morphological characters are listed in Figure 1. Taxonomic sample based on RADseq analysis (except for R23, R24, R25, and R26 identification according to Bailey & Wisskirchen, 2004). Ash, apex shape; INL, internode length; LAL, leaf apex length; LBA, leaf base angle; LBL, leaf blade length; LSh, leaf shape; MLW, maximum leaf width; Nect, nectaries number; PL, petiole length; SDi, stem diameter; St.Color, stem coloration; VBA, vein base angle.

extrafloral Nect was nearly constant for *R.jap*, with only one nectary per node (on the pulvinus), while the other two taxa had at least one supplementary nectary (on the node and the internode).

For distinguishing all taxa studied, the most suitable criteria retained ($R^2 > 0.3$) were LBA ($R^2 = 0.46$), LAL ($R^2 = 0.49$), LSh ($R^2 = 0.41$), Ash ($R^2 = 0.34$), LBL ($R^2 = 0.35$), and also Nect (R^2 not estimated). Concerning *R.jap* and *R.x.boh*, the variables LBA ($R^2 = 0.52$) and Nect (R^2 not estimated) showed the most discriminant traits differentiating these two taxa. Indeed, the extrafloral Nect was relatively fixed for *R.jap* with one nectary by node on the pulvinus.

4 Discussion

4.1 Genetic identity: A knot of hybrids

The overlapping morphological variations have frequently caused taxonomic confusion when trying to distinguish among the taxa within the Asian knotweed complex, especially between *R.x.boh* and *R.jap* (Vuković et al., 2019). During the field sampling of this study, we encountered a challenge in confidently distinguishing between these two taxa based on qualitative observations, while the distinction of *R.sac* remained clear. This morphological perplexity between *R.x.boh* and *R.jap* has led to the underestimation of *R.x.boh* occurrence (Kim & Park, 2000; Mered'a et al., 2019). To avoid this confusion, our genetic analysis

detected the hybrid genotypes of *R.x.boh* using admixture analysis including parental genotypes of *R.jap* and *R.sac*.

The occurrence of only one genotype for *R.jap* in our study corroborates with the literature describing the same pattern (or very low diversity genetic) for this species in Europe (Hollingsworth & Bailey, 2000; Mandák et al., 2005; Bzdega et al., 2016). Often named as the “British clone,” *R.jap* is sometimes compared with “Pando,” the forest of *Populus tremuloides* with the dominance of a single genotype (Grimsby et al., 2007; DeWoody et al., 2008; Groeneveld et al., 2014). Previous genetic studies suggest that all individuals of *R.jap* originate from the same genotype, having propagated by vegetative reproduction (Hollingsworth & Bailey, 2000; Mandák et al., 2005). However, different genotypes are found, proving evidence of sexual reproduction (Forman & Kesseli, 2003). In Belgium, *R.jap* has been observed to engage in reproduction with *R.x.boh*., and this sexual reproduction yields a small percentage of seeds that can be dispersed. This dispersion can allow the formation of genetically differentiated individuals (Tiébré et al., 2007b). Moreover, some populations are known to produce large amounts of seeds with high germination rates (Forman & Kesseli, 2003; Bram & McNair, 2004). In the United States, such introduced populations display higher genetic diversity, demonstrating that the genetic diversity of this species is contingent on the geographical area of introduction (Weston et al., 2005; VanWallendael et al., 2021).

In contrast, the literature has delineated the presence of different genotypes within *R.x.boh* populations in Europe (Pyšek et al., 2003; Mandák et al., 2005; Schnitzler & Bailey, 2008). Our genetic analysis distinguished a single genotype per population, which seems to form two groups with different allelic compositions in the admixture analysis. Both in Europe and in the United States, *R.x.boh* primarily reproduces through vegetative propagation, but it also produces viable seeds (Forman & Kesseli, 2003; Bímová et al., 2004). In the admixture analysis, it is noteworthy that the ancestral genome characteristic of *R.sac* predominates in the genotypes of *R.x.boh*. This can be biologically explained by introgression events with *R.sac*, leading to an increase in its representation in the introgressed genotypes (Tiébré et al., 2007a). Besides, *R.sac* was overrepresented in RADseq analysis, with 14.5 million reads compared to 1.2–6.9 million reads for the other two taxa during Illumina sequencing. This is leading to the prominent identification of its alleles within the hybrid genomes and can be explained by its smaller genome increasing depth coverage. Additionally, a total of more than 100 000 loci were recovered for the *R.sac* sample, as opposed to an average of 38 000 loci recovered for the other samples. However, we found similar results with a 2M-subsample of the *R.sac* sample (data not shown).

Only one *R.sac* sample was used in the RADseq analysis, which did not permit investigation of the genetic diversity for this species. *R.sac* is known to show various genotypes in Europe due to multiple introduction events (Mandák et al., 2003, 2005). The diversity is further enriched by the potential for sexual reproduction and genetic mixing within this species. In the United States, genomic diagnostics revealed the presence of both interspecific and intraspecific sexual reproduction, along with multiple introgressions involving parent species (Grimsby et al., 2007).

4.2 Ploidy levels and taxonomic overlaps

As already mentioned, the three Asian knotweeds present three cytotypes: tetraploid, hexaploid, and octoploid. In our study, in the absence of chromosome counting, we deduced the distinct cytotypes based on the nuclear DNA content determined by flow cytometry. We could distinguish three different ploidy levels, with high congruence with previous literature on *Reynoutria* cytotypes (Suda et al., 2010; Mered'a et al., 2023). Regarding *R.jap*, our 11 populations were estimated as showing an octoploid cytotype in agreement with the literature mentioning *R.jap* as predominantly octoploid (Mandák et al., 2003; Mered'a et al., 2019), and rarely hexaploid (Bzdega et al., 2016; Mered'a et al., 2023), while tetraploid cases are considered as doubtful (Suda et al., 2010; Mered'a et al., 2023). For *R.sac*, the three cytotypes are described with a predominance of tetraploid (Suda et al., 2010; Mered'a et al., 2019), and likewise two cytotypes, that is, tetraploid and hexaploid, were distinguished in our *R.sac* samples. Finally, for *R.x.boh*, the three cytotypes are existing with a predominance of hexaploid (Suda et al., 2010), and some decaploids and aneuploids (Mered'a et al., 2019). For the case of tetraploid *R.x.boh*, Mandák et al. (2003) suggested that this hybrid is derived from *R.sac* and *Reynoutria japonica* var. *compacta* and this particular cytotype is very difficult to differentiate by morphology. Accordingly, in our analysis, a greater variability

of DNA contents was observed for the *R.x.boh* samples, supporting the aneuploid cytotypes described by Schnitzler & Bailey (2008), for the same taxon, in the same region (NE France), as well as in some close localities (R9 and R14, along the Fecht river).

Backcrossing is reported by Tiébré et al. (2007a) in Europe for these species. Moreover, backcrossing does not automatically lead to infertility. Furthermore, an infertile patch can survive and disperse over long distances, as vegetative reproduction is mainly used and can further disperse new ploidy levels in nature. Therefore, with the lack of genetic analysis for all *R.sac* populations and the lack of chromosome counting, this study of the ploidy level has limitations.

4.3 Resolving morphological distinction

Our study offers an opportunity to challenge morphological characters previously documented in the literature using a genetically identified sampling of plants, cultivated under the same common garden environment to limit environmental variability and plasticity. Our results showed that, generally, the leaf of *R.jap* is truncate, ovate with an apex less pronounced, while the leaves of *R.x.boh* and *R.sac* are cordate with an apex strongly pronounced for *R.sac*, supporting previous observations in Europe (Beerling et al., 1994; Bailey & Wisskirchen, 2004; Bailey et al., 2009). Concerning leaf apex criteria, the Asian knotweeds do not show any distinction in Slovakia (Mered'a et al., 2019). Our results showed that the leaves of *R.sac* are the longest and this difference of length was found in several previous studies (Bailey & Wisskirchen, 2004; Bailey et al., 2009; Mered'a et al., 2019), but none of the taxa studied seems to have a significantly different width. For the width, *R.sac* is often presented as the species with the widest leaves in European and Asian populations, but we do not find this peculiarity (Kim & Park, 2000; Bailey et al., 2009; Mered'a et al., 2019). Concerning the extrafloral nectaries criteria, generally, *R.jap* have only one at the pulvinus and the other two taxa may have additional nectaries on their nodes. Bailey & Wisskirchen (2004) have shown the same result but in their observations, additional nectaries are by pairs, while we could observe additional solitary nectaries. Finally, for the stem coloration, a density gradient of red spots is observed: *R.sac* without spots or very rare spots, *R.x.boh* slightly spotted, and *R.jap* with reddish spots or densely redbrown spotted. These observations are in agreement with the results of Beerling et al. (1994) and Bailey & Wisskirchen (2004), except for *R.sac*, which is presented as never having red spots.

Morphological observations revealed a high variability within the same species and for a given character. This phenotypic variation can often be attributed to environmental factors (Beerling et al., 1994). Furthermore, our observations occasionally diverge from the findings of previous research, raising the possibility of distinct geographical morphotypes. Moreover, for *R.x.boh*, the different hybrid groups may also show different phenotypes, as seen in the Czech Republic (Bímová et al., 2004). This phenotypic variation may lead to morphological overlaps between *R.jap* and *R.x.boh* (Mered'a et al., 2019), further complicating taxonomic differentiation, especially with the presence of *R.x.boh*. While foliar criteria can

often be robust, they may fall short in effectively identifying *R.jap* and *R.x.boh* (Mered'a et al., 2019). Other criteria, not tested in our study, have been suggested to differentiate the species, such as the leaf indumentum (Mered'a et al., 2019), cuticular striation (Desjardins et al., 2023), or leaf texture and appearance (Bailey et al., 2009). Another criterion, trichome type of the leaf underside (Pashley, 2003; Bailey et al., 2009), was tested in field and ex situ surveys, but it was not included within the morphometric analysis due to the difficulty of observation. The aim was to propose some easily observable criteria without specific materials and comprehensible to nonspecialists. These criteria were not included in the present study, as they may not always be readily available for quick identification by environmental managers. In response to these challenges, we propose an easy-to-use identification key (Key S1) based mainly on the length of leaf and apex, the shape of the leaf, base, and apex, and the extrafloral nectaries, setting out the exceptions, and that can be applied when evaluating multiple ramets within a studied population, while maintaining a critical perspective.

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Author Contributions

Cybill Staentzel and Laurent Hardion planned and designed the research; Enzo Jugieau and Victor Talmot conducted field and laboratory work on DNA extraction and flow cytometry monitored by Sandra Noir; Enzo Jugieau recovered morphological data; Laurent Hardion analyzed genetic data; Enzo Jugieau and Laurent Hardion performed statistical analysis; Enzo Jugieau wrote the manuscript; and Enzo Jugieau, Victor Talmot, Cybill Staentzel, Sandra Noir, and Laurent Hardion contributed critically to the drafts and gave final approval for publication.

Conflict of Interest

The authors have no conflicts of interest to declare.

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Supplementary Material

The following supplementary material is available online for this article at <http://onlinelibrary.wiley.com/doi/10.1111/jse.13075/supinfo>:

Fig. S1. Histogram of the distribution of genetic distances to delineate the different multilocus genotypes (clone delimitation) based on the maximal distances between two replicates, and using *bitwise.dist()* and *mlg.filter()* functions

from *adegenet* and *poppr* R-packages (Jombart & Ahmed, 2011; Kamvar et al., 2014).

Fig. S2. Photographs of the extrafloral nectaries character studied and its variability between the three species.

Key S1. Determination key for *Reynoutria sachalinensis*, *Reynoutria x bohemica*, and *Reynoutria japonica* var. *japonica*.

Table S1. Sampling information, geographical coordinates, including ploidy level, and genetic results.