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Evaluation of genetic diversity of Himalayan balsam (*Impatiens glandulifera* Royle) populations using microsatellites

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Abstract

Himalayan balsam (*Impatiens glandulifera* Royle) belongs to invasive alien species of European Union concern. Acquisition of the new molecular data from a geographical point of view might be valuable getting ideas about the nowadays invasion steps of *I. glandulifera* to more northern and eastern parts of the Europe, the source population(s) in the local scale as well as for elucidation of the patterns of the spread of alien species in Lithuania. In the period of 2010–2019, 95 sites of *I. glandulifera* were recorded in Lithuania. The present study was aimed at evaluation of genetic diversity at microsatellite loci of Lithuanian populations of *I. glandulifera*. For molecular analysis employing 9 microsatellite (simple sequence repeats, SSR) markers, a total of 20 Lithuanian populations of *I. glandulifera* (15 individuals in each) were used. Allelic richness (A_R) ranged from 1.1 to 1.39 per population, and the expected heterozygosity (H_E) ranged from 0.10 to 0.39 per one. No significant correlation (according to Mantel test) between the geographic distances and genetic differentiation was determined between the Lithuanian populations of *I. glandulifera*. AMOVA showed that variability within the populations (56.2%) was higher than that among the populations (43.7%). According to the principal coordinate analysis (PCoA), three principal axes explained 60% of the total genetic variation of populations. Significant results were obtained when the populations were grouped according to the five geographical areas of Lithuania: variation among the groups comprised 5.73% of the total variation. Bayesian clustering analysis indicated the highest ΔK values at K = 12, and the next highest value was K = 3. It may indicate the multiple introductions of *I. glandulifera* to Lithuania.

Keywords: Balsaminaceae, simple sequence repeats, SSR, molecular markers, invasion, alien species.

Introduction

The Balsaminaceae family includes two genera: *Hydrocera* and *Impatiens*, one of which (*Impatiens*) has close to 1 000 species (Yu et al., 2016). *Impatiens* spp. are morphologically diverse in their flower structure including species with large flowers of bright colours (Kim et al., 2015; Janssens et al., 2022). Therefore, it is not surprising that some *Impatiens* species are cultivated worldwide as bedding and potted plants (Luo et al., 2020). Among the species of *Impatien*s there are ornamental plants of global economic importance that grow well in the temperate climate of Europe. Nowadays, the most important species of ornamental *Impatiens* are *I. hawkeri* (Samiei et al., 2018) and *I. waleriana* (Wang et al., 2018) in addition to the well-known for a long time *I. balsamina* and *I. glandulifera* (Power, Sánchez Vilas, 2020).

Introduction of *I. balsamina* and *I. glandulifera* to non-native areas of their distribution have caused ecological consequences such as occupying semi-natural and natural habitats after the escape from the gardening (Power, Sánchez Vilas, 2020). These adverse consequences of introduction are particularly evident in *I. glandulifera*, which is currently better known worldwide as invasive of wide geographical range species rather than an ornamental plant (Coakley, Petti, 2021). *I. glandulifera* was listed as an invasive alien species of European Union concern in the 2017 update to Regulation (EU) No. 1143/2014 (EU, 2017).

Due to an extensive character of invasions, *I. glandulifera* is well known in many countries with a variety of synonyms of the species common name such as Indian balsam, ornamental jewelweed, policeman's helmet, bobby tops, copper tops, gnome's hatstand, Himalayan balsam, and kiss-me-on-the-mountain, and in internationally agreed titles such as *Impatiens roylei* Walp. It is among the tallest annual plants in Europe, forming 2 × 5–4 cm long flowers resembling lips with

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an upper hood-like petal, able to hold even a bumblebee foraging for nectar and a reminder of petals forming a big enough area convenient for landing of insects-pollinators. In addition to a great variation in descriptions of flower colour (Janssens et al., 2022), it should be mentioned that the flowers of all individuals of some studied by us populations were the same colour, while the colour of the flowers of individuals of the other populations was very diverse: white, pale pink, pink, rose, violet, and dark purple (personal observations of Lithuania sites). A variety of the flower flavonoids have been determined (Vieira et al., 2016). *I. glandulifera* is widespread along various habitats including forests (Čuda et al., 2017), ruderal places and rivers, rivulars, roadside ditches, and other type watercourses as prevailing sites (Power, Sánchez Vilas, 2020).

Escape of *I. glandulifera* in Poland as the southern neighbour of Lithuania was recorded in 1890 (Tokarska-Guzik, 2005 and others cited therein) with the geometric increase in numbers of the individuals up to the end of the former century. Similar time (1898) of the escape of *I. glandulifera* from cultivation was dated in Latvia as the northern neighbour of our country (Priede, 2009). Such data together with the facts of Kew Botanical Gardens about less than a 20-year period required between introduction and liberation of the species shows that *I. glandulifera* may have been introduced in the gardens of Lithuania earlier than it was officially stated for the first time (Dagys et al., 1934). The species did not appear in the list of adventive plants of Lithuania at the time when *I. parviflora* has been already mentioned (Natkevičaitė-Ivanauskienė, 1951). Very soon in the Manual for Identification of Plants of Lithuania (Snarskis, 1954) *I. glandulifera* was described as grown in the gardens and cemeteries, frequently found as escape close to the sites of cultivation.

Despite years of *I. glandulifera* research, this plant is among the most intensively investigated aliens at present (Tanner, Gange, 2020; Helsen et al., 2021). The assessment of the role of *I. glandulifera* in the plant community by planting or uprooting has demonstrated that removal might cause an increase in the growth of *Urtica dioica* and an elevation of the mass of local neighbouring grasses (Bieberich et al., 2018). Effects of *I. glandulifera* on fungal and bacterial community (Gaggini et al., 2018) as well as other impacts on multiple components of the ecosystem are under investigation (Davis et al., 2018; Kiełtyk, Delimat, 2019). Till now, *I. glandulifera* remains as the alien species with unresolved yet methods for eradication and the development of methods for eradication is in progress (Leblanc, Lavoie, 2017; Oliver et al., 2020). Mass spectroscopy of acid-soluble proteins allowed one to distinguish several types of *I. glandulifera* that differ in the sensitivity to *Puccinia komarovii* var. *glanduliferae*.

I. glandulifera is not as harmful as some other aliens of Europe like *Fallopia japonica* (Čuda et al., 2017; Coakley, Petti, 2021). Due to the toleration of wide range edaphic and aerial conditions, application of *I. glandulifera* to remediate polluted media is considered (Van Meerbeek et al., 2015; Coakley et al., 2019), and other applications in biotechnology are discussed (Klančnik, 2021). Also, the value of secondary metabolites for human diseases treatment is investigated (Orzelska-Górka et al., 2019).

For explanation of invasion sources and drivers as well as for successful applications in biotechnology, knowledge in the molecular biology of wider geographical range populations of *I. glandulifera* is required. Acquisition of the new molecular data from a geographical point of view might be valuable getting ideas about the nowadays invasion steps of *I. glandulifera* to more northern and eastern parts of the Europe and the source population(s) in the local scale as well as for elucidation of the patterns of spread within the species range in Lithuania. After the development of microsatellite markers specific to *I. glandulifera* (Provan et al., 2007; Walker et al., 2009), numerous studies have concentrated on intercontinental and regional patterns of *I. glandulifera* invasion (Love et al., 2013; Hagenblad et al., 2015; Nagy, Korpelainen, 2015). In contrast, surprisingly little information is available about genetic features of invasive plants at the local scale.

Following our analyses of populations of that species by some dominant markers (Zybartaite et al., 2011; Kupcinskiene et al., 2015), in the present study, the aim was to evaluate the genetic diversity at microsatellite loci of the Lithuanian populations of *I. glandulifera*.

Material and methods

Sampling sites. The same populations of *Impatiens glandulifera* were selected as in the previous study (Zybartaite et al., 2011). To reflect geographical ranges of *I. glandulifera* in Lithuania, populations were selected through all the country and, if possible, close to the borders. The populations were titled according to the geographical names of their location, and the names of populations were abbreviated by three letters (Figure 1).

Rok – Rokiškis, AMa – Anykščiai-Malgažatavas, Jon – Jonava, VVi – Vilnius-Visoriai, VSn – Vilnius-Šnipiškės, VFa – Vilnius-Fabijoniškės, KAS – Kaunas-A.Šančiai, KMa – Kaunas-Marvelė, KLa – Kaunas-Lampėdžiai, KZa – Kaunas-Žaliakalnis, Gir – Girininkai, Vai – Vaišvydava, Jie – Jieznas, Kru – Kruonis, VaZ – Varėna-Žiūrai, Rau – Raudondvaris, Bel – Belvederis, Jur – Jurbarkas, Pal – Palanga, Juo – Juodkrantė

Figure 1. Sampling locations of the Lithuanian populations of *Impatiens glandulifera*

Characteristics of habitats. For each site, several habitat features were recorded: water source proximity (an overmoistured site; parallel to the dike/ditch/ stream; 50–100 m from the river bank; no water basin in the vicinity); light intensity estimated by the life form of neighbouring plants (an open place without shrubs/separate trees; near shrubs/separate trees; park/forest edge); traffic intensity/ road vicinity (along the blacktop road with an intensive traffic; along the blacktop road of the city/town with a low intensity traffic; along the road without blacktop in the forest; no road/path in the vicinity); geographical subdivision into five areas of Lithuania: Kaunas district, southern part of Lithuania, Vilnius city, northern part of Lithuania, and western part of Lithuania.

Extraction of DNA. Approximately 0.1 g of fresh plant material was ground in liquid nitrogen, and the total DNA was extracted by a DNA Purification Kit #KO512 (Thermo Fisher Scientific Baltics, Lithuania); details have been described by Kupcinskiene et al. (2015). The concentration and purity of DNA samples were determined spectrophotometrically with a NanoDrop 2000 (Thermo Fisher Scientific, USA) and by agarose gel (1.5%) electrophoresis. For molecular analyses, the concentration of DNA of each sample was adjusted approximately to 20 ng μl^{-1}.

Simple sequence repeats (SSR) analysis. Six primer pairs developed by Provan et al. (2007) and three by Walker et al. (2009) were used (Table 1). Polymerase chain reaction (PCR) was performed in a final volume of 12.5 µl containing 40 ng genomic DNA, 6.25 µl 2x PCR Master Mix (0.05 U μl^{-1} Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0,4 mM each dNTP) (Thermo Fisher Scientific Baltics), and 5 pmol μl^{-1} of each primer (Thermo Fisher Scientific). Reverse primers were labelled with fluorescent dyes. Loci IGNSSR101, IGNSSR104, IGNSSR106, IGNSSR203, IGNSSR210, and IGNSSR240 were amplified according to the PCR conditions described by Provan et al. (2007), and loci A2, A3, and A21 amplification was done according to Walker et al. (2009). The PCR product (its mixture with formamide and LIZ-500 DNA size standard) was denaturated for 3 min at 95°C and detected using a 3100 Genetic Analyzer (Thermo Fisher Scientific). Several loci in the same run have been analysed. The 1st combination consisted of primers IGNSSR101 (PET), IGNSSR210 (NED), IGNSSR240 (VIC), and A2 (6-FAM), and the 2nd one included IGNSSR104 (VIC), IGNSSR106 (PET), IGNSSR203 (6-FAM), A3 (VIC), and A21 (NED). In the 2nd combination, two primers labelled with the same dye were used because their generated DNA fragments differed markedly in the number of base pairs. Individuals were genotyped manually using the software GeneMapper, version 4.0 (Thermo Fisher Scientific).

Table 1. Characteristics of 9 microsatellite loci used in the analysis of the Lithuanian populations of *Impatiens glandulifera*

Locus	Locus ID	Fluorescence label	Primer sequence (5'→3')	Allele size range bp	Number of allele
IGNSSR101[a] EF025990	101	PET	ACGACAAGCGGAGTCATTCT AAGAAAGCACGGCAGAGAGT	101–107	3
IGNSSR104[a] EF025992	104	VIC	CCACCATACCTTCTTCTCCTG GTTGCCCGGAAGTAGACATT	109–123	6
IGNSSR106[a] EF025993	106	PET	CCTGTTCATATTCAGACCCAAA ATAATTGCATGCCCCCATT	117–135	5
IGNSSR203[a] EF025994	203	6-FAM	CAAAGGGCGACGGTTTCT TTCCATGGACAATTCCTTCA	142–148	3
IGNSSR210[a] EF025995	210	NED	CCAGAGAGGTGGAGGTTCAA GAAAGCAGGTTCCGTCGATA	120–129	2
IGNSSR240[a] EF025997	240	VIC	CGGCTTCTGATTCACGAAAT TGCTAACCGGATTCTTCTGG	137–155	4
A2F[b] A2R	A2	6-FAM	ACCACGGACGCAAGTGA GCAAGAGAAGTTGGCGGAA	310–334	6
A3F[b] A3R	A3	VIC	ACTTCCATGTGTTATTGA TGAAAGATGGGTTACATT	350–352	2
A21F[b] A21R	A21	NED	ACTCTTCTGGCTAAGCTG AAAGCGAGAAGTTGGCG	339–355	7

Markers labelled [a] were developed by Provan et al. (2007), [b] – by Walker et al. (2009)

Statistical analysis. To test each population for the presence of null alleles, for SSR data, program FreeNA was used (Chapuis et al., 2008). The expected (H_E) and observed (H_O) heterozygosity, pairwise genetic differentiation coefficient (F_{ST}), and molecular variance among and within populations (AMOVA) were calculated using the software Arlequin, version 3.5.1 (Excoffier, Lischer, 2010). Additionally, the hierarchical AMOVA subdivision of populations according to their habitat features was performed. The allelic richness (A_R), inbreeding coefficient (F_{IS}), and F_{ST} were calculated by the package FSTAT, version 3.9.3.2 (Goudet, 2002). To calculate significant deviations from the Hardy-Weinberg equilibrium (HWE), the software GENEPOP, version 4.2 (Rousset, 2008) was used. The Mantel test (used to assess correlations between genetic differentiation and geographic distances) and the principal coordinate analysis (PCoA) were performed by the software GenAlEx, version 6.501 (Peakall, Smouse, 2012). Based on genetic distances, the unweighted pair group method with the arithmetic mean (UPGMA) dendrogram was constructed by the program TFPGA, version 1.3 (Miller, 1997) for the bootstrap analysis performing 1 000 iterations. The Bayesian clustering analysis of population structure was carried out using the software Structure, version 3.2.4 (Hubisz et al., 2009). More details for statistical analysis were described earlier (Anderson et al., 2018; Jocienė et al., 2022; Krokaitė et al., 2022).

Results

Twenty Lithuanian populations of *I. glandulifera* were examined according to 9 microsatellite loci. A total of 38 alleles over all loci were identified and ranged from 2 (loci 210 and A3) to 7 (locus A21). The allele size ranged from 101 to 355 bp in length (Table 1). Each population at each locus was checked for the presence of null alleles. The total number of marker/population combinations was 180 (20 populations and 9 markers; data not shown); in 122 cases, the frequency of null alleles was less than 5%. All loci were included in the dataset because potential null alleles occurred in different loci and populations.

The total number of alleles per population (N_t) ranged between 12 (populations VaZ and Juo) and 24 (population KMa), the mean number being 18 (Table 2). One private allele (N_p) was detected for the following 5 populations: Rok, Jon, VSn, KAS, and Rau. The mean number of alleles per locus per population (N_a) ranged between 1.33 (populations VaZ and Juo) and 2.67 (population KMa), the mean number for all populations being 2. The allelic richness (A_R) ranged from 1.1 (population VaZ) to 1.39 (population KMa), the mean value being 1.27. The mean value of percentage of polymorphic loci (P%) was 65%. The least polymorphic populations were VaZ and Juo (33%), and the most one was KLa (100%). The observed heterozygosity (H_O) ranged from 0.08 (populations VVi and Vai) to 0.29 (population Jon), the mean value for all populations being 0.22. The expected heterozygosity (H_E) ranged from 0.10 (population VaZ) to 0.39 (population KMa), the mean value for all populations being 0.28. The inbreeding coefficient (F_{IS}) values ranged from −0.26 (population Rok) to 0.47 (population Jon), the mean value for all populations being 0.17. Four populations (KMa, KLa, Vai, and Pal) showed a significant deviations from HWE.

The coefficient of pairwise genetic differentiation (F_{ST}) between the Lithuanian populations of *I. glandulifera* ranged in the interval of 0.02–0.84 (Table 3). The lowest genetic differentiation was observed between the VVi and Jur populations, whereas the highest was observed between the Jon and KZa ones.

Table 2. Genetic diversity parameters for the 20 Lithuanian populations of *Impatiens glandulifera* based on 9 microsatellite loci

Population	N_t	N_p	N_a	A_R	P%	H_O	H_E	F_{IS}
Rok	15	1	1.67	1.18	44.4	0.23	0.18	−0.26
AMa	15	0	1.67	1.27	55.6	0.27	0.27	−0.02
Jon	16	1	1.78	1.15	44.4	0.08	0.15	0.47
VVi	22	0	2.44	1.35	88.9	0.29	0.35	0.19
VSn	19	1	2.11	1.25	77.8	0.17	0.26	0.33
VFa	19	0	2.11	1.31	88.9	0.21	0.31	0.34
KAS	21	1	2.33	1.35	66.7	0.28	0.36	0.20
KMa	24	0	2.67	1.39	77.8	0.23	0.39	0.40*
KLa	22	0	2.44	1.36	100.0	0.26	0.36	0.29*
KZa	18	0	2.00	1.22	55.6	0.22	0.22	0.01
Gir	15	0	1.67	1.24	55.6	0.26	0.24	−0.04
Vai	19	0	2.11	1.35	66.7	0.29	0.35	0.19*
Jie	18	0	2.00	1.28	55.6	0.24	0.28	0.14
Kru	19	0	2.11	1.32	66.7	0.22	0.32	0.31
VaZ	12	0	1.33	1.10	33.3	0.10	0.10	−0.05
Rau	16	1	1.78	1.25	66.7	0.28	0.24	−0.14
Bel	18	0	2.00	1.31	66.7	0.25	0.31	0.19
Jur	20	0	2.22	1.32	77.8	0.21	0.36	0.34
Pal	20	0	2.22	1.33	77.8	0.19	0.33	0.41*
Juo	12	0	1.33	1.13	33.3	0.12	0.13	0.09
Mean	18	0.2	2.00	1.27	65.0	0.22	0.28	0.17

Explanations of the abbreviations of the populations under Figure 1; N_t – total number of alleles per population, N_p – private number of alleles, N_a – mean number of alleles per locus per population, A_R – allelic richness, P% – percentage of polymorphic loci, H_O – observed heterozygosity, H_E – expected heterozygosity, F_{IS} – inbreeding coefficient; * – differences significant at $p \leq 0.05$ from HWE according to Fisher test

Table 3. Pairwise genetic differentiation coefficient (F_{ST}) of the Lithuanian populations of *Impatiens glandulifera* based on 9 microsatellite loci

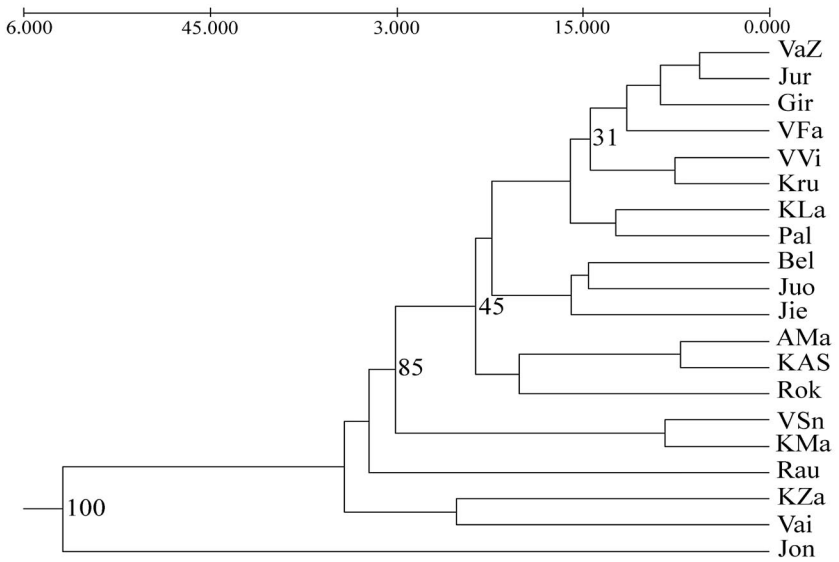
Pop	Rok	AMa	Jon	VVi	VSn	VFa	KAS	KMa	KLa	KZa	Gir	Vai	Jie	Kru	VaZ	Rau	Bel	Jur	Pal	Juo
Rok	x																			
AMa	*0.24*	x																		
Jon	*0.72*	*0.67*	x																	
VVi	*0.38*	*0.29*	*0.54*	x																
VSn	*0.70*	*0.52*	*0.81*	*0.61*	x															
VFa	*0.49*	*0.37*	*0.56*	*0.08*	*0.63*	x														
KAS	*0.12*	*0.11*	*0.63*	*0.22*	*0.55*	*0.27*	x													
KMa	*0.46*	*0.33*	*0.61*	*0.33*	*0.24*	*0.36*	*0.29*	x												
KLa	*0.34*	*0.23*	*0.63*	*0.07*	*0.56*	*0.11*	*0.13*	*0.26*	x											
KZa	*0.67*	*0.54*	*0.84*	*0.50*	*0.79*	*0.41*	*0.36*	*0.48*	*0.38*	x										
Gir	*0.52*	*0.43*	*0.50*	*0.07*	*0.75*	*0.24*	*0.39*	*0.51*	*0.32*	*0.70*	x									
Vai	*0.41*	*0.32*	*0.74*	*0.43*	*0.71*	*0.40*	*0.23*	*0.46*	*0.31*	*0.45*	*0.57*	x								
Jie	*0.42*	*0.31*	*0.59*	*0.24*	*0.47*	*0.29*	*0.26*	*0.22*	*0.18*	*0.49*	*0.44*	*0.42*	x							
Kru	*0.51*	*0.46*	*0.44*	*0.08*	*0.71*	*0.11*	*0.37*	*0.45*	*0.26*	*0.61*	*0.05*	*0.53*	*0.37*	x						
VaZ	*0.44*	*0.38*	*0.64*	*0.10*	*0.68*	*0.33*	*0.33*	*0.44*	*0.24*	*0.69*	*0.17*	*0.60*	*0.37*	*0.26*	x					
Rau	*0.52*	*0.43*	*0.71*	*0.40*	*0.66*	*0.40*	*0.34*	*0.40*	*0.35*	*0.49*	*0.54*	*0.45*	*0.42*	*0.50*	*0.52*	x				
Bel	*0.36*	*0.33*	*0.72*	*0.37*	*0.62*	*0.44*	*0.15*	*0.33*	*0.28*	*0.40*	*0.55*	*0.46*	*0.34*	*0.52*	*0.46*	*0.45*	x			
Jur	*0.22*	*0.18*	*0.52*	*0.02*	*0.51*	*0.14*	*0.09*	*0.25*	*0.04*	*0.43*	*0.18*	*0.35*	*0.18*	*0.18*	*0.09*	*0.30*	*0.23*	x		
Pal	*0.34*	*0.12*	*0.66*	*0.12*	*0.50*	*0.20*	*0.13*	*0.26*	*0.06*	*0.49*	*0.38*	*0.39*	*0.22*	*0.35*	*0.22*	*0.36*	*0.31*	*0.04*	x	
Juo	*0.37*	*0.43*	*0.81*	*0.56*	*0.73*	*0.63*	*0.35*	*0.47*	*0.46*	*0.75*	*0.72*	*0.51*	*0.44*	*0.68*	*0.65*	*0.62*	*0.43*	*0.42*	*0.53*	x

Note. Explanations of the abbreviations of the populations under Figure 1; values in italic are significant at $p \leq 0.05$, the number of permutations is 1 000.

Calculated from genetic distances, the UPGMA clustering analysis revealed Jon as the most distinct population (Figure 2). The rest populations have splitted into two clusters. The first cluster consisted of KZa and Vai, the second one comprised the rest populations with the most distinct of them populations Rau and KMa, and, also, VSn.

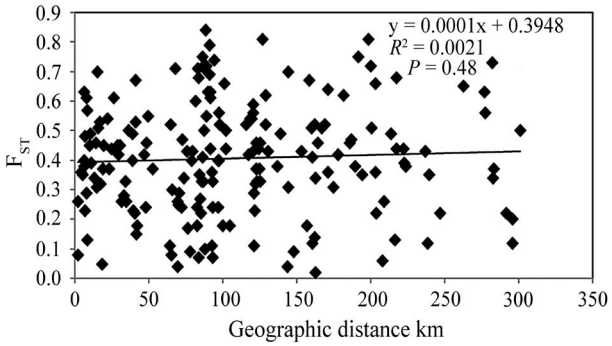
According to Mantel test, no significant correlation between the geographic distances and genetic differentiation was detected between the populations of *I. glandulifera* (Figure 3).

Habitat features of populations were characterised (Table 4). According to the water source, the biggest part of populations (9) was situated in the sites with no water basin in the vicinity, and overmoistured soil was characteristic of one population only. According to the light intensity, the biggest part of populations (12) was situated near the shrubs/separate trees, and the least common habitat for populations (3) was park/forest edge. According to the traffic intensity or road vicinity, 7 populations grew in the site with no road/path in the vicinity, and the minimum number of populations grew along the blacktop road with a low intensity traffic. According to the geographical subdivision into five areas of Lithuania, the biggest part of populations (6) was situated in Kaunas district, and the minimum number of populations (3 in each area) was situated in Vilnius city and the northern part of Lithuania.



Note. The scale represents the genetic distances; bootstrap values (% of 1 000 replicates) are shown above the branches; only values above 30 are presented; explanations of the abbreviations of the populations under Figure 1.

Figure 2. Dendrogram of genetic relationships among 20 Lithuanian populations of *Impatiens glandulifera* using the UPGMA algorithm and the genetic distances based on 9 microsatellite loci



Note. Labels are pairwise values of genetic differentiation coefficient (y axis) and geographic distance (x axis).

Figure 3. Relationships between the genetic differentiation coefficient (F_{ST}) and geographic distance among 20 Lithuanian populations of *Impatiens glandulifera* based on 9 microsatellite loci

The populations were grouped according to the features of habitats, and the mean of polymorphic loci percent was calculated. According to the water source, the most polymorphic were the populations situated in a 50–100 m distance from the river bank (72.2%); according to the light intensity, the most polymorphic populations were situated in the park/forest edge (68.5%); according to the traffic intensity or road vicinity, the most polymorphic populations grew along the blacktop road with a low intensity traffic (74.1%); according to the geographical subdivision into five areas of Lithuania, the most polymorphic populations were situated in Vilnius city (85.2%).

AMOVA showed that the variability within all 20 populations (56.2%) was higher than among the populations (43.7%); the coefficient of genetic differentiation (F_{ST}) was 0.44 (Table 5).

The hierarchical AMOVA among the groups of populations subdivided according to the habitat features has shown that in most cases the differentiation was very low and non-significant (Table 5). Significant results were obtained only in one case when the populations were grouped according to the five geographical areas of Lithuania, and the variation among the groups comprised 5.73% of the total variation; also, the variation within the populations was higher than among the populations within groups: 55.70% and 38.56%, respectively.

According to the principal coordinate analysis (PCoA), three principal axes explained 60% of the total genetic variation: 27.3% by the 1st principal axis and

Table 4. Characteristics of the site of Lithuanian populations of *Impatiens glandulifera*

Population	Water source proximity (1)	Light intensity (2)	Traffic intensity (3)	Geographical subdivision (4)
Rok	1–2	2–2	3–3	4–4
AMa	1–4	2–1	3–3	4–4
Jon	1–3	2–2	3–1	4–4
VVi	1–4	2–2	3–3	4–3
VSn	1–4	2–2	3–2	4–3
VFa	1–2	2–2	3–4	4–3
KAS	1–2	2–2	3–3	4–1
KMa	1–4	2–1	3–1	4–1
KLa	1–3	2–3	3–4	4–1
KZa	1–4	2–2	3–1	4–1
Gir	1–4	2–1	3–3	4–2
Vai	1–1	2–2	3–1	4–1
Jie	1–2	2–1	3–4	4–2
Kru	1–2	2–2	3–4	4–2
VaZ	1–4	2–1	3–4	4–2
Rau	1–4	2–2	3–2	4–1
Bel	1–2	2–3	3–1	4–5
Jur	1–4	2–2	3–2	4–5
Pal	1–2	2–2	3–4	4–5
Juo	1–2	2–3	3–4	4–5

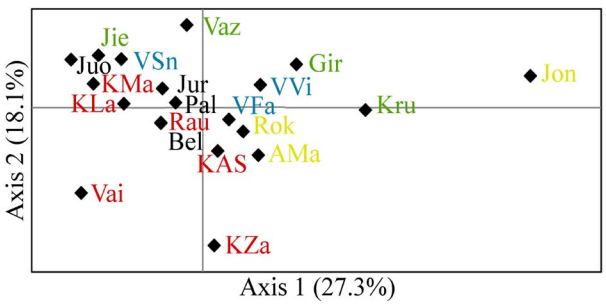
Explanations of the abbreviations of the populations under Figure 1; water source proximity (1–1 – overmoistured site, 1–2 – parallel to the dike/ditch/ stream, 1–3 – 50–100 m from the river bank, 1–4 – no water basin in the vicinity); light intensity estimated by the life form of neighbouring plants (2–1 – open place without shrubs/separate trees, 2–2 – near shrubs/separate trees, 2–3 – park/forest edge); traffic intensity/road vicinity (3–1 – along the blacktop road with an intensive traffic, 3–2 – along the blacktop road of the city/town with a low intensity traffic, 3–3 – along the road without blacktop in the forest, 3–4 – no road/path in the vicinity); geographical subdivision into five areas of Lithuania (4–1 – Kaunas district, 4–2 – southern part of Lithuania, 4–3 – Vilnius city, 4–4 – northern part of Lithuania, 4–5 – western part of Lithuania)

Table 5. Analysis of the molecular variance (AMOVA) of Lithuanian populations of *Impatiens glandulifera* based on 9 microsatellite loci

Source of variation	df	SS	Variance components	Percentage of variation	*F* statistic	*P*
Variance assuming two hierarchical levels						
Among populations	19	260.19	0.44	43.74	$F_{ST} = 0.44$	0.00
Among individuals within populations	580	326.60	0.56	56.26		0.00
Total	599	586.79	1.00			
Variance assuming three hierarchical levels						
Water source						
Among groups of populations	3	44.55	0.01	1.09	$F_{CT} = 0.01$	0.36
Among populations within groups	16	215.65	0.43	42.85	$F_{SC} = 0.44$	0.00
Among individuals within populations	580	326.60	0.56	56.05	$F_{IS} = 0.44$	0.00
Total	599	586.79	1.00			
Light intensity						
Among groups of populations	2	24.74	−0.01	−0.89	$F_{CT} = -0.01$	0.56
Among populations within groups	17	235.46	0.44	44.42	$F_{SC} = 0.44$	0.00
Among individuals within populations	580	326.60	0.56	56.47	$F_{IS} = 0.44$	0.00
Total	599	586.79	1.00			
Traffic						
Among groups of populations	3	42.46	0.01	0.37	$F_{CT} = 0.00$	0.43
Among populations within groups	16	217.74	0.43	43.41	$F_{SC} = 0.44$	0.00
Among individuals within populations	580	326.60	0.56	56.22	$F_{IS} = 0.44$	0.00
Total	599	586.79	1.00			
Proximity to buildings						
Among groups of populations	2	32.84	0.02	1.54	$F_{CT} = 0.02$	0.25
Among populations within groups	17	227.35	0.43	42.46	$F_{SC} = 0.43$	0.00
Among individuals within populations	580	326.60	0.56	56.00	$F_{IS} = 0.44$	0.00
Total	599	586.79	1.01			
Geographical subdivision into five areas of Lithuania						
Among groups of populations	4	76.33	0.06	5.73	$F_{CT} = 0.06$	0.04
Among populations within groups	15	183.86	0.39	38.56	$F_{SC} = 0.41$	0.00
Among individuals within populations	580	326.60	0.56	55.70	$F_{IS} = 0.44$	0.00
Total	599	586.79	1.01			

Note. df – degree of freedom, SS – sum of squares, *P* – significance of differences; coefficients of differentiation: F_{ST} – among populations; F_{CT} – among groups of populations, F_{SC} – among populations within groups, F_{IS} – among individuals within populations; details for habitat features are provided in Table 4.

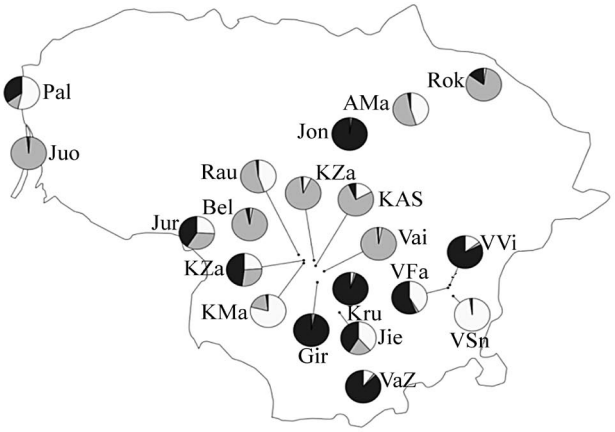
18.1% by the 2nd one (Figure 4). In the PCoA plot, the most distinct populations were Jon, KZa, and Vai; the rest populations were located close to each other. The results of PCoA were similar to those of UPGMA clustering analysis. PCoA did not allocate the populations into subgroups based on the subdivision into five areas of Lithuania.



Explanations of the abbreviations of the populations under Figure 1; colours representing the geographical subdivision into five areas of Lithuania: red – Kaunas district, green – southern part of Lithuania, blue – Vilnius city, yellow – northern part of Lithuania, black – western part of Lithuania

Figure 4. Principal coordinate analysis (PCoA) of the genetic distances between 20 Lithuanian populations of *Impatiens glandulifera* based on 9 microsatellite loci

The Bayesian clustering analysis indicated that the highest *ΔK* values were obtained at K = 12; the next best value was K = 3. Three clusters were selected as better revealing the research data (Figure 5).



Note. Explanations of the abbreviations of the populations under Figure 1; three different colours (white, grey, and black) of the circles represent part of each out of three genetic clusters in each population.

Figure 5. Genetic structure of the Lithuanian populations of *Impatiens glandulifera* according to the Bayesian clustering analysis

The populations Jon (black colour cluster), Juo (grey colour cluster), and VSn (white colour cluster) were exceptionally affiliated to a single cluster. One out of three genetic clusters was prevailing for Bel and Vai (grey colour cluster), and Kru and Gir (black colour cluster). The populations Pal, Jur, KLa, and Jie showed an admixed clustering pattern consisting of similar parts of all three genetic clusters.

Discussion

In 1988–1995, Gudžinskas and Sinkevičienė (1995) have recorded 43 sites of *I. glandulifera* in Lithuania. In 2010–2019, we have recorded 6 sites of *I. glandulifera*. Similar findings, 134 sites of *I. glandulifera*, were observed in Latvia (in 4.8% of 2783 quadrats scored) by Priede (2009). Before the present study, in Lithuania, few population assessments of herbaceous plant species have employed microsatellite markers (Anderson et al., 2018; Vyšniauskienė et al., 2020). For invasive in Lithuania *Impatiens* spp., only dominant markers have been used (Zybartaite et al., 2011; Kupcinskiene et al., 2015; Krokaitė et al., 2022).

According to microsatellite loci, the populations of *I. glandulifera* were analysed within the natural (Asia) and invasive ranges of distribution in Western Europe (Nagy, Korpelainen, 2015; Hagenblad et al., 2015) but Baltic States. Nagy and Korpelainen (2015) have analysed 12 populations from the invasive range (170 individuals from Canada, Finland, and the United Kingdom) and 9 populations from the native regions (34 individuals from India and Pakistan). Hagenblad et al. (2015) analyses encompassed 10 populations from the invasive range (299 individuals from Norway, Sweden, Germany, Belgium, and France) and 3 populations from the native regions (79 individuals from India). As in our case, in other studies (Provan et al., 2007; Walker et al., 2009; Love et al., 2013; Helsen et al., 2019), only populations of *I. glandulifera* from the invasive range in Europe were analysed. Provan et al. (2007) have analysed one population from Wales and one from Ireland (total 20 individuals); Walker et al. (2009) investigated 13 populations from North England (total 390 individuals); Love et al. (2013) have analysed 9 populations from Wales and 10 populations from Ireland (total 15–35 individuals from each population); Helsen et al. (2019) investigated 13 populations from North France, Belgium, Germany, South Sweden, Central Sweden, and Central Norway (299 individuals). In the current study, previously analysed by RAPD markers, 20 populations from Lithuania were examined (300 individuals) (Zybartaite et al., 2011). In addition, using RAPD and inter simple sequence repeat (ISSR) markers, four Lithuanian populations (AMa, Juo, Vai, and VaZ, total 60 individuals) of *I. glandulifera* were compared to four populations from Czech Republic (Kupcinskiene et al., 2015).

Concerning *I. glandulifera* populations, all studies differed in the number and set of microsatellite markers employed. For *I. glandulifera* analysis, Provan et al. (2007) have used 8 microsatellite markers developed by them; Walker et al. (2009) have developed 3 new microsatellite markers and used only these. Later, such set of personally developed microsatellite markers was supplemented by the sets of other researchers (Walker et al., 2009; Love et al., 2013). Nagy and Korpelainen (2015) have used the same microsatellite marker set like Love et al. (2013). Hagenblad et al. (2015) have used 6 out of 8 microsatellite markers developed by Provan et al. (2007) and 3 developed by Walker et al. (2009). Helsen et al. (2019) have used the same marker set like Hagenblad et al. (2015). In the current study, 6 out of 8 markers developed by Provan et al. (2007) and all 3 markers developed by Walker et al. (2009) were employed (Table 1). In Hagenblad et al. (2015), Helsen et al. (2019) and our studies, the number of used microsatellite markers was the same, only the set of markers was different. In the current study, marker 213 failed to amplify, and in the cases of Hagenblad et al. (2015) and Halsen et al. (2019), marker 106 failed to amplify. Marker 103 was not amplified by three different groups of investigators. In our opinion, this could have happened due to an insufficient optimisation of PCR conditions or due to different geographic scales of the studies. Alleles of 103 microsatellite loci could have been lost during the expansion of invasion from the United Kingdom to the continental Europe. Something similar might have happened with the alleles of 213 loci in the Lithuanian populations. *I. glandulifera* could have come to Lithuania not only from the United Kingdom and Western Europe, but also by other routes.

Beside the difference in methodology (geography, the number of populations and individuals assessed, and the set of microsatellite markers), all researchers of *I. glandulifera* populations differed in various parameters discussed. In the case of Walker et al. (2009), 13 populations at 3 microsatellite loci had 36 alleles; in our case, 20 populations at the same loci (A2, A3, and A21) had 15 alleles (Table 1). In the study of Provan et al. (2007), 2 populations at 6 microsatellite loci had 18 alleles, and in our case, according to the same loci (101, 104, 106, 203, 210, and 240), 32 alleles were recorded.

The average number of alleles per population from the invasive range was 17 in the study of Hagenblad et al. (2015), and in the current study, it was 18. The number of private alleles ranged in the interval of 0–2 (Hagenblad et al., 2015); one private allele was detected in 20% of the populations, and two private ones were detected in 10% of the populations. For the case of Lithuanian populations, the percent was similar: one private allele was documented in 25% of the populations.

Only one study was distinguished by the high mean number of alleles (3.2) per locus per population (Nagy, Korpelainen, 2015). In the study of Hagenblad et al. (2015) and in our case, the values were lower and similar to each other, 1.9 and 2.0, respectively (Table 2). The mean values for allelic richness (A_R) over all loci ranged in the interval of 1.36–2.53 for the populations from Wales and Ireland (Love et al., 2013). For the Lithuanian populations, this value was of a smaller size and ranged from 1.10 to 1.39. Differences might occur due to an unequal number of individuals, populations, and distinct geography of population sampling.

For the Lithuanian populations, the observed heterozygosity (H_O) values (0.09–0.28) were the most similar to the values (0.07–0.25) obtained from the populations in Norway, Sweden, Germany, Belgium, and France (Hagenblad et al., 2015). Meanwhile, the expected heterozygosity (H_E) values (0.10–0.39) of Lithuanian populations were the most similar to the values of populations (0.12–0.41) from Wales and Ireland (Love et al., 2013). In the current study, the H_O values in 3 out of 20 populations (15%) were higher than the H_E ones: in 14 out of 20 populations (70%). This ratio was lower, and in 3 out of 20 populations (15%) these parameters were equal.

I. glandulifera is a facultative allogamous plant pollinated by insects (by bumblebees especially) (Bartomeus et al., 2010) and self-pollinated (Helsen et al., 2021). The plant is geitonogamous but not cleistogamous (Clements et al., 2008). The environment of each population might differ in various peculiarities including diversity and abundance of insect species.

A significant positive inbreeding coefficient was observed in the four populations of Lithuania: KMa, KLa, Vai, and Pal; it indicates the lack of heterozygous individuals, which might be caused by self-pollination. In the study of Norway, Sweden, Germany, Belgium, and France (Hagenblad et al., 2015), a significant positive inbreeding coefficient was estimated in 7 out of 10 populations within the invasive range, and in the case of the later performed investigation by the same research (Helsen et al., 2019), a positive inbreeding coefficient was estimated in 5 out of 13 populations. The majority of Lithuanian populations had a high (>0.20) inbreeding coefficient; the same was true for the earlier examined other European populations of this species (Walker et al., 2009; Hagenblad et al., 2015; Nagy, Korpelainen, 2015; Helsen et al., 2019). Genetic differentiation between populations might become higher when repeated or multiple introductions occur. Differences between the Lithuanian populations of *I. glandulifera* ranged in the interval of 0.02–0.84 and were proximate to that (0.06–0.73) estimated by Hagenblad et al. (2015).

According to the UPGMA and PCoA of genetic distances (Figures 2 and 4), the Lithuanian populations of *I. glandulifera* did not split according to a geographical location. Similar findings were observed by the other authors (Walker et al., 2009; Hagenblad et al., 2015), where only a few geographically close populations were located next to each other in the dendrogram (Walker et al., 2009), and PCoA revealed that individuals of Europe populations were overlapped (Hagenblad et al., 2015).

Significant correlations (according to Mantel test) were not observed between the geographic and genetic distances of Lithuanian populations (Figure 3); the same was true in the study of Walker et al. (2009). No significant correlation was observed for some other invasive species (Marrs et al., 2008; Gaudeul et al., 2011). A low significant correlation between the genetic and geographical distances was observed for the *Arundo donax* L. populations invasive in the United States (Tarin et al., 2013). Molecular variance within the Lithuanian populations of *I. glandulifera* was higher than among the populations (56%) and was similar (62–65%) to that (Table 5) obtained by Hagenblad et al. (2015).

The hierarchical AMOVA based on habitat features was only significant when the populations were subdivided into five geographical areas of Lithuania: Kaunas district, southern part of Lithuania, Vilnius city, northern part of Lithuania, and western part of Lithuania. The Bayesian clustering analysis revealed that the Lithuanian populations might be grouped into 12 or 3 clusters (Figure 5). At the same time, it indicated that the populations might have got their origin from three different sources. Two populations, Juo and VSn, situated near the borders of Lithuania, were exclusively affiliated to a single cluster coloured in grey and white, respectively (Figure 1). Near the port located population Juo might have been introduced from Kaliningrad (Russia) or any other country and later could have spread to the Central Lithuania by the river Nemunas or transported from city to city, because the majority of populations affiliated to this cluster belong to Central Lithuania sites. The population VSn might have been introduced from Belarus or Poland. The third population affiliated to a single cluster was Jon (Figure 4, coloured in black). To that site *I. glandulifera* might have been introduced from Latvia and later could have spread to the South of Lithuania.

The Bayesian clustering analysis of *I. glandulifera* populations revealed the presence of three genetic clusters. Such finding was similar to the other assessments performed in Western Europe by Nagy and Korpelainen (2015) and Hagenblad et al. (2015). Native and invasive populations of *I. glandulifera* were grouped into the three clusters. The first cluster was exclusively composed of the populations from the native range (India and Pakistan); the second cluster consisted of the populations from the United Kingdom and Canada, and one population was from Finland; the third cluster was composed of the rest populations from Finland (Nagy, Korpelainen, 2015). In the other study (Hagenblad et al., 2015), populations from the native and invasive range were also grouped into three clusters: one cluster was exclusively composed of populations from Kashmir, another consisted of more southern European populations (France, Belgium, Germany, and South Sweden), and the last one consisted of more northern European populations (Central Sweden and Central Norway). In both cases, neither populations from Finland (Nagy, Korpelainen, 2015) nor those from Sweden (Hagenblad et al., 2015) did not group into the same cluster, but in both cases the natural range populations formed a separate cluster.

The data obtained by our research together with the former assessments of other scientific groups suggest the necessity of extension of investigation geography within the invasive range distribution of *I. glandulifera*.

Conclusions

1. Genetic diversity of the Lithuanian populations of *Impatiens glandulifera*, measured as the expected heterozygosity (H_E) values, was low and in agreement with the other country studies of the populations of *Impatiens glandulifera* within the invasive range.

2. Population grouping into five geographic areas revealed a significant differentiation; in the case of habitat features, a significant differentiation was not documented.

3. The Bayesian analysis revealed three clusters of the Lithuania populations of *I. glandulifera*; it may indicate the multiple introductions of *I. glandulifera* to Lithuania.

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Bitinės sprigės (*Impatiens glandulifera* Royle) populiacijų genetinė įvairovė pagal mikrosatelitų lokusus

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Santrauka

Bitinė sprigė (*Impatiens glandulifera* Royle) priklauso susirūpinimą keliančioms Europos Sąjungos invazinėms rūšims. Geografiniu atžvilgiu naujų molekulinių duomenų gavimas gali būti naudingas, siekiant įgyti žinių apie šių dienų *I. glandulifera* invazijos kelius į šiaurines bei rytines Europos dalis ir vietinio masto invazinių populiacijų šaltinius, taip pat norint nustatyti svetimkraščių rūšių plitimo Lietuvoje dėsningumus. Lietuvoje 2010–2019 m. buvo aptiktos 95 *I. glandulifera* paplitimo vietos. Tyrimo tikslas – įvertinti Lietuvos *I. glandulifera* populiacijų mikrosatelitinių lokusų genetinę įvairovę. Iš viso, panaudojus 9 mikrosatelitų (paprastų kartotinių sekų, angl. SSR) pradmenų poras, molekulinė analizė atlikta su 20 Lietuvos *I. glandulifera* populiacijų (po 15 individų kiekvienoje). Alelių gausa vienai populiacijai svyravo nuo 1,1 iki 1,39, o tikėtinas heterozigotiškumas – nuo 0,10 iki 0,39 populiacijai. Lietuvos *I. glandulifera* populiacijoms reikšmingos koreliacijos tarp geografinių atstumų ir genetinės diferenciacijos (Mantelio testu) nenustatyta. Molekulinės įvairovės analizė (angl. AMOVA) parodė, kad kintamumas visų 20 populiacijų viduje tarp individų (56,2 %) buvo didesnis nei tarp populiacijų (43,7 %). Remiantis principinių koordinačių analize (PCO), trys pagrindinės ašys paaiškino 60 % viso genetinio populiacijų kintamumo. Populiacijas sugrupavus pagal penkias Lietuvos geografines vietoves, nustatyta reikšminga populiacijų diferenciacija, o kintamumas tarp grupių sudarė 5,73 % bendro kintamumo. Bajeso analizė didžiausias ΔK vertes parodė esant K = 12, o kita didžiausia vertė buvo K = 3.
Remiantis *I. glandulifera* populiacijų įvairovės tyrimo duomenimis, Lietuvoje bitinė sprigė galėjo būti introdukuota keletą kartų.

Reikšminiai žodžiai: Balsaminaceae, paprastosios kartotinės sekos, SSR, molekuliniai žymekliai, invazija, svetimkraštės invazinės rūšys.