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Identification of alien *Fallopia* taxa using molecular methods

Določanje tujerodnih dresnikov (*Fallopia* spp.) z molekulskimi metodami

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Abstract: The non-native species of knotweeds (*Fallopia* sect. *Reynoutria*) are morphologically very similar and it is often difficult to distinguish between the hybrid *F.* ×*bohemica* and parental taxa, *F. japonica* and *F. sachalinensis*. To distinguish 30 samples of knotweeds, collected in Slovenia, we used PCR RFLP analysis of the *trn*K intron of plastid DNA in combination with the amplification of microsatellite nuclear locus KW6, which is a specific diagnostic marker for *F. sachalinensis*. We established that the combination of both markers unambiguously identifies the following samples: *F. japonica* (var. *japonica*), *F. sachalinensis* and *F.* ×*bohemica.* Based on described molecular markers we confirmed that the maternal parent of the taxon *F.* ×*bohemica* was *F. japonica* for all analysed hybrids. In addition, two species from *Fallopia* sect. *Sarmentosae* (*F. baldschuanica* and *F. multiflora*) were also analysed. Both could be distinguished from species of *Fallopia* sect. *Reynoutria,* but for the discrimination between them, some other markers should be used.

Keywords: *Fallopia*, invasive species, Slovenia, hybridization, plastid DNA, *trn*K, microsatellite locus, KW6, RFLP

Izvleček: Tujerodni dresniki (*Fallopia* sect. *Reynoutria*) so si morfološko zelo podobni. Še posebej težko je razlikovati med križancem *F.* ×*bohemica* in starševskima vrstama *F. japonica* in *F. sachalinensis*. Z uporabo dveh različnih molekulskih označevalcev, plastidnega zaporedja DNA *trn*K v kombinaciji z analizo RFLP in mikrosatelita KW6, ki je potencialno specifičen za vrsto *F. sachalinensis*, smo analizirali 30 vzorcev dresnikov, ki smo jih nabrali na območju Slovenije. Ugotovili smo, da s kombinacijo obeh označevalcev nedvoumno določimo naslednje taksone: *F. japonica* (var. *japonica*), *F. sachalinensis* in *F.* ×*bohemica.* S pomočjo molekulskih označevalcev smo kot materinsko vrsto vseh vzorcev križancev določili japonski dresnik. V analizo smo vključili tudi predstavnika slakovcev, *F. baldschuanica* in *F. multiflora*. Ta se s pomočjo uporabljenih molekulskih označevalcev zanesljivo razlikujeta od dresnikov, za razlikovanje med vrstama slakovcev pa bi bilo treba poiskati nove označevalce.

Ključne besede: *Fallopia*, invazivna vrsta, Slovenija, križanci, plastidna DNA, *trn*K, mikrosatelitni lokus, KW6, RFLP

Introduction

Invasive alien taxa from genus *Fallopia* origin in East-Asia and are one of the most troublesome invaders worldwide, especially in Europe and North America (Bailey et al. 2009, Tiebre et al. 2007, Forman and Kesseli 2003). The influence of invasive alien species is known as the second greatest threat to biodiversity after the loss of habitats (Wilson 1991) and this is probably one of the main reasons for numerous studies of invasive species in last decades.

In Slovenia, three alien taxa from *Fallopia* sect. *Reynoutria* known also as knotweeds, thrive: *F. japonica* (Houtt.) Ronse Decr. var. *japonica*, *F. sachalinensis* (F. Schmidt) Ronse Decr., and their hybrid *F. ×bohemica* (Chrtek & Chrtková) J. P. Bailey (Strgulc Krajšek and Jogan 2011). Additionally, two deciduous vining woody perennials from *Fallopia* sect. *Sarmentosae* have been reported, *F. baldschuanica* (Regel) Holub (Strgulc Krajšek and Jogan 2011) and *F. multiflora* (Thunb.) Haraldson (Balant et al. 2015).

In Europe, *F. japonica* is a male sterile clone (Bailey et al. 2009) and is reproducing vegetatively. Hollingsworth and Bailey (2000) analysed 150 British and 16 other European populations of *F. japonica* and determined identical RAPD profiles for all samples, thus proving clonal growth. In native range of East Asia, *F. japonica* occurs also as male (rarely) and hermaphroditic plants, but they were not introduced to Europe (Bailey 2003). *Fallopia japonica* in Europe is octoploid (2n=8x=88) (Bailey et al. 2007), as was proved also by measuring the genome size of samples in Czech Republic (Suda et al. 2010) and in Slovenia (Strgulc Krajšek and Dolenc Koce 2015). In Slovenia the species is common in lowlands but present also in higher regions up to 1150 m a. s. l. in Julian Alps (Strgulc Krajšek and Jogan 2011).

Fallopia sachalinensis is a tetraploid (2n=4x=44), represented in Europe with hermaphroditic and female plants (Bailey et al. 2009). Hexaploid and octoploid specimens can be found in introduced range (Czech Republic) too, but they are very rare (Mandak et al. 2003). In Slovenia it is known from less than 20 localities scattered across the country (Strgulc Krajšek and Jogan 2011).

The hermaphroditic plants of *F. sachalinensis* are the source of pollen, and in localities where they grow close to *F. japonica*, hybrids (*F. ×bohemica*) may occur. The hybrids have been known from English gardens since at least 1872 (Bailey and Conolly 2000). They have the highest genetic variation of all taxa belonging to *Fallopia* sect. *Reynoutria* because hybrids originated multiple times (Mandak et al. 2005) and they can backcross to their parents (Bailey et al. 2009). Hybrids of F1 generation are mostly hexaploids (2n=6x=66) but many other chromosome numbers have been reported, mostly among different backcrosses (Bailey et al. 2009). The flowers of *F. ×bohemica* in Slovenia are male or hermaphroditic, both types on the same plant (Strgulc Krajšek and Jogan 2011).

The reliable determination of the taxa from the *Fallopia* sect. *Reynoutria* can be difficult, especially when completely developed leaves or flowers are not available. Two specific molecular markers were developed for *F. japonica* and *F. sachalinensis.*

The first is the *trn*K intron of plastid DNA, which has been used extensively as a phylogenetic marker for classification of plants. In identification of *Fallopia* taxa it has been used in combination with restriction endonuclease HhaI that cuts the intron in two segments of different size in *F. japonica* var. *japonica* (Hollingsworth et al. 1999), whereas in *F. sachalinensis* this specific restriction site is lacking. Plastids within the study group are inherited maternally and unidirectional hybridisation between *F. japonica* var. *japonica* and *F. sachalinensis* has been proved for specimens collected in Great Britain, always with *F. japonica* var. *japonica* as the maternal parent (ibid.).

The second marker is nuclear, simple sequence repeat (SSR) or microsatellite locus, KW6, which is potentially diagnostic and specific marker for *F. sachalinensis* (Grimsby et al. 2007).

Plant material of different *Fallopia* taxa, collected in West and Central Europe (Tiebre et al. 2007, Hollingsworth et al. 1999, Hollingsworth and Bailey 2000, Suda et al. 2010) and North America (Gammon et al. 2007, Grimsby et al. 2007, Forman and Kesseli 2003, Grimsby and Kesseli 2010) have been already examined in studies dealing with sexual reproduction of knotweeds, but there is a lack of the knowledge about the South European populations. We used PCR

RFLP analysis of the *trn*K intron in combination with amplification of nuclear, microsatellite locus KW6. The combination of both markers could be used for the recognition of hybrids between *F. japonica* and *F. sachalinensis* (Grimsby et al. 2007, Hollingsworth et al. 1999) and for the determination of female parent species of *F. ×bohemica* specimens. We have included also the samples of *F. baldschuanica* and *F. multiflora* to inspect if these two species can be distinguished from taxa of *Fallopia* sect. *Reynoutria* using the same molecular markers.

Material and methods

Plant material and DNA extraction

A total of 32 samples of *Fallopia* taxa were collected in different sites across Slovenia (Appendix): 8 samples of *F. japonica*, 2 samples of *F. sachalinensis,* 20 hybrid plants (*F. ×bohemica*), 1 specimen of *F. baldschuanica* and 1 of *F. multiflora*.

In previous study (Bímová et al. 2003) was shown that regeneration from rhizomes was the major mode of vegetative reproduction in the complex of *Fallopia* sect. *Reynoutria*, therefore rhizome segments with some winter buds were collected in the field. They were further grown in the laboratory in plastic pots (12 L) filled with garden substrate, watered, and only slightly pressed into the soil. After the development of young shoots, some intact leaves were removed and 100 mg of fresh young intact leaf material was used for DNA extraction with a Plant Genomic DNA Miniprep Kit according to recommended protocol (Sigma-Aldrich).

RFLP analysis of the trnK intron

The *trn*K intron was amplified using the universal primers described by Demesure et al. (1995). All PCRs were done in 25 µL with 2 µL diluted genomic DNA (approximately 20 ng), 1 µL of each primer (10 µM), 2.5 µL of 10X reaction buffer, 2.5 µL of 25 mM $MgCl_2$, 0.6 µL of 10 mM combined dNTPs, and 0.2 µL of Taq DNA polymerase (5 units/µL). The remaining volume was filled up with water. Amplification was performed with the following cycles: 5 min denaturation at 94 °C; 30 cycles of 92 °C for 45 s, 53 °C for 45 s, 72 °C for 3 min; followed by a final extension at 72 °C for 10 min. The PCR products were then digested with restriction endonuclease HhaI as follows: 5 µL of PCR product was digested with 1 µL HhaI (10 units/µL) for 1 hour at 37 °C. Results of restriction were visualized on a 1 % agarose gels (0.5X TBE buffer) stained with ethidium bromide under the ultra violet light.

Amplification of the KW6 SSR

The nuclear, simple sequence repeat (SSR) marker KW6 was amplified using primers as described in Grimsby et al. (2007). All PCRs were done in 25 µL with 2 µL diluted genomic DNA, 1 µL of each primer (10 µM), 2.5 µL of 10X reaction buffer, 2.5 µL of 25 mM $MgCl_2$, 0.6 µL of 10 mM combined dNTPs, and 0.2 µL of Taq DNA polymerase (5 units/µL). The remaining volume was filled up with water. The PCR profile was: 1 cycle of 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 54 °C, and 30 s at 72 °C and finally 1 cycle at 72 °C for 10 min. PCR products were separated on 1.7 % agarose gels (0.5X TBE buffer) and visualized with ethidium bromide under the ultra violet light.

Results and discussion

The combination of all results of the molecular analysis of *Fallopia* samples is shown in Table 1 and on the photo of electrophorese gel (Fig. 1). The result for *F. multiflora* sample was the same as for *F. baldschuanica* and is not shown.

Table 1: Results of the RFLP analysis of the *trn*K intron and amplification of microsatellite locus KW6 in *Fallopia* samples from Slovenia.
Tabela 1: Rezultati analize RFLP introna *trn*K in pomnožitve mikrosatelitskega lokusa KW6 pri slovenskih vzorcih iz rodu *Fallopia*.

	RFLP analysis of the *trn*K intron		KW6 amplification
Taxon	**Amplification of plastid (trnK) DNA**	**Digestion with the restriction enzime HhaI**	
F. japonica	2 700 bp long fragment	1 600 in 1 100 bp long restriction fragments	not amplified
F. sachalinensis	2 700 bp long fragment	undigested 2 700 bp long fragment	338 bp long fragment
F. ×bohemica	2 700 bp long fragment	1 600 in 1 100 bp long restriction fragments	338 bp long fragment
F. baldschuanica	2 700 bp long fragment	undigested 2 700 bp long fragment	not amplified
F. multiflora	2 700 bp long fragment	undigested 2 700 bp long fragment	not amplified

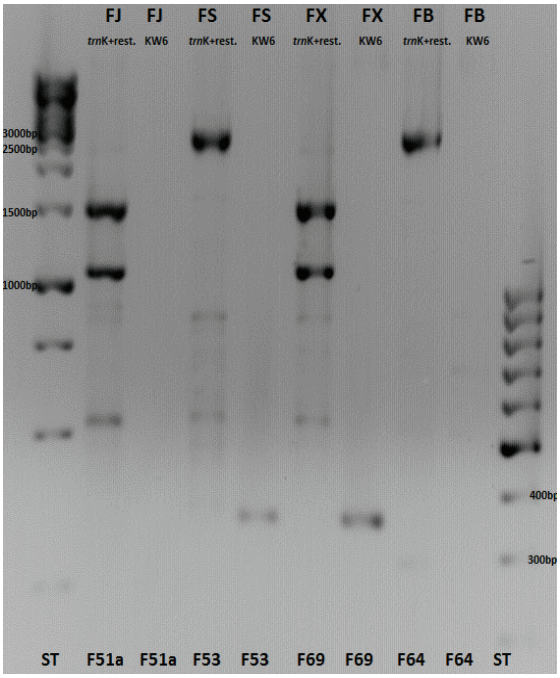

Figure 1: PCR RFLP profiles of *trn*K intron of cpDNA (*trn*K + restriction) and profiles of PCR products of microsatellite locus KW6 nDNA (KW6) of knotweeds *F. japonica*, *F. sachalinensis*, hybrid *F. ×bohemica* and Russian vine (*F. baldschuanica*).
Legend: ST – 1 kbp size marker (left) and 100bp size marker (right); FJ – *F. japonica*; FS – *F. sachalinensis*; FX – *F. ×bohemica*; FB – *F. baldschuanica*. Marks at the bottom of the gel represent sample ID.
Slika 1: PCR RFLP profili introna *trn*K cpDNA (*trn*K + restrikcija) in profili produktov pomnoževanja mikrosatelitskega lokusa KW6 nDNA (KW6) dresnikov *F. japonica*, *F. sachalinensis,* križanca *F. ×bohemica* ter grmastega slakovca (*F. baldschuanica*).
Legenda: ST – DNA standard z lestvico 1.000 bp (skrajno levo) oz. 100 bp (skrajno desno); FJ – *F. japonica*; FS – *F. sachalinensis*; FX – *F. ×bohemica*; FB – *F. baldschuanica*. Oznake v spodnjem delu slike so oznake vzorcev.

The plastid sequence *trn*K is 2700 bp long and has been amplified in all analyzed specimens of *F. japonica, F. sachalinensis*, *F. ×bohemica*, *F. baldschuanica,* and *F. multiflora*. After the digestion with the enzyme HhaI, it remained intact in *F. sachalinensis, F. baldschuanica* and *F. multiflora.* In *F. japonica* and *F. ×bohemica,* it was cut in two restriction fragments, 1600 and 1100 base pairs long; all analysed hybrid specimens *F. ×bohemica* had plastid haplotype of *F. japonica.* Given the maternal inheritance of plastid DNA in *Fallopia* (Hollingsworth et al. 1999) and the apparent male sterility of *F. japonica* in Slovenia (Strgulc Krajšek and Jogan 2011) the present results provide strong evidence that hybridization between *F. japonica* and *F. sachalinensis* in investigated samples from Slovenia was unidirectional, with *F. japonica* as the maternal parent. The unidirectional hybridisation has been previously demonstrated in Great Britain (Hollingsworth et al. 1999).

The nuclear, simple sequence repeat (SSR) marker, KW6 was amplified in all samples of *F. sachalinensis* and *F. ×bohemica.* It is a potentially diagnostic *F. sachalinensis*-specific marker (Grimsby et al. 2007). Its presence in hybrid specimens together with restricted sequence *trn*K confirms the morphological identification of analysed specimens.

Since the hybrid *F. ×bohemica* is highly fertile (Tiebre et al. 2007, Strgulc Krajšek and Dolenc Koce 2015) and the plants mostly have male flowers (Grimsby et al. 2007, Strgulc Krajšek and Dolenc Koce 2015), backcrosses may occur (Bailey et al. 2009). Among such backcrosses the genomic marker KW6 for *F. sachalinensis* can be lost and hybrid can no longer be identified (Grimsby and Kesseli 2010). In our study there were no such specimens.

We have tested the selected markers also for *F. baldschuanica* and *F. multiflora*. The combination of these results was different than for taxa from *Fallopia* sect. *Reynoutria*. The plastid *trn*K segment was not digested by enzyme HhaI (as in *F. sachalinenis*) and there was no amplification of KW6 marker (as in *F. japonica*). Consequentely the combination of these markers could be used for the differentiation between the sections, but not between the species within *Fallopia* sect. *Sarmentosae* (*F. baldschuanica* and *F. multiflora*).

Another hybrid between *F. japonica* and *F. baldschuanica*, *F. ×conolyana* J. P. Bailey, is also expected in Slovenia, as it was reported from many European countries (Bailey 2001). In Great Britain it is common along railways (ibid.). In the costal part of Slovenia where *F. baldschuanica* is invasive (Strgulc Krajšek and Jogan 2011) and in some localities in other parts of the country where it is grown as an ornamental plant in gardens (Balant et al. 2015), the hybrid *F. ×conolyana* could occur. This hybrid could not be distinguished from *F. japonica* with molecular markers we have used.

In conclusion, (1) we established that the combination of both markers (PCR RFLP analysis of the *trn*K intron of plastid DNA and the amplification of microsatellite nuclear locus KW6) unambiguously identifies *F. japonica* (var. *japonica*), *F. sachalinensis* and *F. ×bohemica*, (2) we confirmed that *F. japonica* was the maternal parent of all analysed hybrid *F. ×bohemica*, and (3) *F. baldschuanica* and *F. multiflora* can be distinguished from species of *Fallopia* sect. *Reynoutria* using the combination of both markers, but for the discrimination between these two species other markers are needed.

Povzetek

V Sloveniji so prisotni trije taksoni invazivnih tujerodnih dresnikov (*Fallopia* sect. *Reynoutria*) in sicer *Fallopia japonica* (Houtt.) Ronse Decr. var. *japonica*, F. *sachalinensis* (F. Schmidt) Ronse Decr. in njun križanec *F. ×bohemica* (Chrtek & Chrtková) J. P. Bailey. Taksoni so si med seboj morfološko zelo podobni, še posebej težko pa je razlikovati med križancem *F. ×bohemica* ter starševskima vrstama *F. japonica* in *F. sachalinensis*.

Z uporabo dveh različnih molekulskih označevalcev, plastidnega zaporedja DNA *trn*K v kombinaciji z analizo RFLP z uporabo restrikcijske endonukleaze HhaI ter mikrosatelita KW6, ki je potencialno specifičen za vrsto *F. sachalinensis*, smo analizirali 30 vzorcev dresnikov, ki smo jih nabrali na območju Slovenije. Med njimi je bilo 8 vzorcev vrste *F. japonica*, 2 vzorca *F. sachalinensis* in 20 vzorcev križanca *F. ×bohemica*.

Ugotovili smo, da lahko s kombinacijo obeh označevalcev nedvoumno določimo vse tri taksone (tab. 1, sl. 1). Pri vrsti *F. japonica* (var. *japonica*) pomnoženi 2700 bp dolg fragment *trn*K endonuk-

leaza HhaI razreže na dva dela velikosti 1600 bp in 1100 bp, mikrosatelit KW6 pa se ne pomnoži. Pri vrsti *F. sachalinensis* pomnoženi 2700 bp dolg fragment *trn*K po uporabi endonukleaze HhaI ostane cel, pomnoži pa se mikrosatelit KW6. Pri križancu *F. ×bohemica* pomnoženi 2700 bp dolg fragment *trn*K endonukleaza HhaI razreže na dva dela velikosti 1600 bp in 1100 bp, kot je značilno za japonski dresnik, pomnoži pa se mikrosatelit KW6, kar je značilno za sahalinski dresnik. S tem smo tudi potrdili, da je japonski dresnik materinska vrsta vseh analiziranih vzorcev križancev.

V analizo smo vključili tudi dva predstavnika slakovcev, *F. baldschuanica* (Regel) Holub in *F. multiflora* (Thunb.) Haraldson. Oba se s pomočjo uporabljenih molekulskih označevalcev zanesljivo razlikujeta od dresnikov (tab. 1, sl. 1), saj pomnoženi 2700 bp dolg fragment *trn*K po uporabi endonukleaze HhaI ostane cel, mikrosatelit KW6 pa se ne pomnoži. Za razlikovanje med vrstama slakovcev pa bi bilo treba poiskati nove označevalce.

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Appendix: Sampling localities

Japanese knotweed (*Fallopia japonica*)

F10–KAMNIK; Slovenia, Gorenjska, Kamnik, Zgornje Perovo, beside petrol station, road branch to Zg. Perovo; N 46°12'34.0'', E 14°36'27.8''; ruderal place beside road; MTB: 9753/4; leg: S. Strgulc Krajšek and S. Anžlovar, 15.4.2010.

F22–KRESNICE; Slovenia, Zasavje, Kresnice, by River Sava; N 46°06'20.3'', E 14°46'44.6''; meadow beside River Sava; MTB: 9854/4; leg: N. Jogan, 27.9.2010.

F27–BF-MOST; Slovenia, Ljubljana, beside Večna pot, by Glinščica brook, beside bridge on eastern side of Biotechnical Faculty; N 46°02'58.3'', E 14°28'33.0''; bank of regulated brook; MTB: 9952/4; leg: S. Strgulc Krajšek and S. Anžlovar, 15.4.2010.

F51–CELJE; Slovenia, Štajerska, Celje, Selce, between brooks Voglajna in Ležnica before the confluence, by the road underpass under railway line, N 46°13'58,68'', E 15°16'42,58''; ruderal place beside road; MTB 9757/4; leg: S. Strgulc Krajšek and T. Pogačnik Lipovec, 22.9.2010.

F59–CELJE-POLULE; Slovenia, Štajerska, Celje, Polule, left bank of river Savinja by road bridge; N 46°13'0,49'', E 15°15'51,02''; river bank; MTB 9757/4; leg: S. Strgulc Krajšek and M. Bjelić, 26.1.2012.

F60–DEBRO-SAVINJA; Slovenia, Štajerska, by the road Celje–Laško, Debro; N 46°10›39,65», E 15°14'19,39'' river bank, MTB 9857/1; leg: S. Strgulc Krajšek and M. Bjelić, 26.1.2012.

F61–MARNO; Slovenia, Štajerska, by the road Rimske Toplice–Hrastnik, E from Marno; N 46°8›18,4», E 15°8'37,67''; forest edge, 9866/4; leg: S. Strgulc Krajšek and M. Bjelić, 26.1.2012.

F65–ZAGORJE; Slovenia, Zasavje, Zagorje, Toplice, left bank of regulated brook Medija; N 46°8›19,98», E 14°59'26,11''; brook bank, MTB 9955/1; leg: M. Bjelić, 25.2.2012.

Giant knotweed (*Fallopia sachalinensis*)

F50–CELJE-POLULE; Slovenia, Štajerska, Celje, Polule, around the bus station opposite the school; N 46°12'58.1'', E 15°15'45.3'', ruderal site beside road; MTB 9757/4; leg.: M. Škornik, 20.6.2010.

F53–CELJE-MEDLOG; Slovenia, Štajerska, Celje, Medlog, beside connecting road from Medlog to highway A1; N 46°14'45.2'', E 15°13'46.0''; road bank; MTB 9757/3; leg: S. Strgulc Krajšek and T. Pogačnik Lipovec, 22.9.2010.

Bohemian knotweed (*Fallopia × bohemica*)

F01–VIŽMARJE; Slovenia, Ljubljana, Vižmarje, by the Tacenska street, N 46°6'22,61", E 14°27'45,31"; ruderal site by the street; MTB: 9852/4, leg: S. Strgulc Krajšek, 11.11.2009.

F02–KOKRICA; Slovenia, Gorenjska, Kranj, Naklo, crossroad on the Kranj to Naklo road to highway A2 (Kranj Zahod); N 46°15'45.3'', E 14°19'60.0''; ruderal place beside road; MTB: 9752/1; leg: S. Strgulc Krajšek, 20.4.2010.

F05–KRANJ; Slovenia, Gorenjska, Kranj, Savski otok, left riverbank of Sava; N 46°14'37.3'', E 14°21'01.0''; river bank; MTB: 9752/3; leg: S. Strgulc Krajšek, 20.4.2010.

F07–MEDVODE; Slovenia, Gorenjska, Medvode, Jeprca, unpaved parking place by Jeprca to Medvode road; N 46°09'15.6'', E 14°23'54.8''; ruderal place beside road resting place; MTB: 9852/1; leg: S. Strgulc Krajšek, 20.4.2010.

F09–ŠENTVID; Slovenia, Ljubljana, Šentvid, Poljane; N 46°05'44.6''; E 14°28'19.9''; ruderal place beside road; MTB: 9952/2; leg: S. Strgulc Krajšek, 20.4.2010.

F24–BF; Slovenia, Ljubljana, beside Večna pot, by Glinščica brook, by fence of Biotechnical Faculty; N 46°03'02.7'', E 14°28'19.5''; bank of regulated brook; MTB: 9952/2; leg: S. Strgulc Krajšek and S. Anžlovar, 15.4.2010.

F34–FUŽINE; Slovenia, Ljubljana, Studenec, by PST trail, 100 m E from the bridge over Ljubljanica river; N 46°3'1,06", E 14°33'57,59"; river bank; MTB: 9953/1; leg: S. Strgulc Krajšek, 15.6.2010.

F35–CHENGDUYSKA; Slovenia, Ljubljana, Fužine, by the bus station Chengdujska; N 46°3'17,21", E 14°34'1,14"; ruderal place beside the road; MTB: 9953/1; leg: S. Strgulc Krajšek, 15.6.2010.

F38–BOKALCE; Slovenia, Ljubljana, Bokalce; N 46°3'1,25", E 14°26'32,7"; by the unpaved field road; MTB: 9952/2; leg: S. Strgulc Krajšek and B. Dolinar, 29.7.2010.

F49–CELJE-BREG; Slovenia, Štajerska, Celje, Breg, by Dornov studenec; N 46°13'20,75", E 15°16'12,74"; ruderal place beside road; MTB: 9757/4; leg: S. Strgulc Krajšek and T. Pogačnik Lipovec, 22.9.2010.

F51–CELJE; Slovenia, Štajerska, Celje, Selce, between brooks Voglajna and Ležnica before the confluence, by the road underpass under railway line, N 46°13'58,68", E 15°16'42,58"; ruderal place beside road; MTB 9757/4; leg: S. Strgulc Krajšek and T. Pogačnik Lipovec, 22.9.2010.

F56–PIRNIČE; Slovenia, central Slovenia, Zgornje Pirniče, by the road from Zg. Pirniče to graveyard; N 46°8'33,98", E 14°25'58,25"; ruderal place beside the road; MTB: 9852/4, leg: S. Strgulc Krajšek, 23.1.2012.

F57–VERJE; Slovenia, central Slovenia, Verje, by the bridge over Sava under the hydroelectric power plant Medvode; N 46°8'38,25", E 14°24'53,34"; ruderal place beside the road; MTB: 9852/4, leg: S. Strgulc Krajšek, 23.1.2012.

F58–MEDVODE-KROŽIŠČE; Slovenija, Gorenjska, Medvode, by regional road Jeprca–Medvode, bus station near roundabout; N 46°8'52,69", E 14°24'30,23"; ruderal place beside the road; MTB: 9852/3; leg: S. Strgulc Krajšek, 23.1.2012.

F66–BRITOF; Slovenia, Gorenjska, Kranj, by the road to Šenčur, 200 m SE from the Britof; N 46°15'37,95", E 14°23'41,18"; ruderal place beside the road; MTB: 9752/1; leg: S. Strgulc Krajšek, 2.3.2012.

F68–BLED-CESTA SVOBODE; Slovenia, Gorenjska, Bled, road resting place by the road to Bohinj, SW from Bled; N 46°21'24,16", E 14°5'26,23"; ruderal place beside road resting place; MTB: 9650/2; leg: S. Strgulc Krajšek, 2.3.2012.

F69–KRANJ-BRDO-AC; Slovenia, Gorenjska, Kranj, by the road Kranj–Kokrica by overpass over highway; N 46°15'45,65", E 14°21'13,47"; ruderal place beside the road; MTB: 9752/1; leg: S. Strgulc Krajšek, 2.3.2012.

F70–NOMENJ(V); Slovenia, Gorenjska, Sava Bohinjka valley, Nomenj, road branch from the main road on the E edge of the settlement; N 46°17'19,41", E 14°0'47,44"; ruderal place beside the road; MTB: 9750/1; leg: S. Strgulc Krajšek, 2.3.2012.

F71–NOMENJ(Z); Slovenia, Gorenjska, Sava Bohinjka valley, Nomenj, branch from the main road on the W edge of the settlement; N 46°17'20,95", E 13°59'51,46"; ruderal place beside the road; MTB: 9750/1; leg: S. Strgulc Krajšek, 2.3.2012.

F72–LJ-BRDO-AC; Slovenia, Ljubljana, Brdo, highway exit Brdo, under the highway overpass; N 46°3'18,41", E 14°27'9,68"; ruderal place beside the road, MTB: 9952/2; leg: S. Strgulc Krajšek and M. Bjelić, 30.3.2012.

Russian vine (*Fallopia baldschuanica*)

F64–NOVA GORICA; Slovenia, Vipava valley, Nova Gorica, by the gas station in Grčna; N 45°57'8,98", E 13°39'8,46"; bushes by the road, MTB: 0047/2; leg: N. Jogan, 3.11.2010.