

RESEARCH ARTICLE

Genetic diversity assessment of bittersweet (*Solanum dulcamara*, *Solanaceae*) germplasm using conserved DNA-derived polymorphism and intron-targeting markers

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Keywords

European native *Solanum* species; gene-targeting markers; genetic resources; late blight resistance; *Phytophthora infestans*; population fragmentation.

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Abstract

Bittersweet (*Solanum dulcamara*), a European native weed, is widespread across a variety of habitats and often occurs as a coloniser of open, disturbed, ephemeral environments or wetlands, although it is also found in mountain habitats and on forest edges. As recent studies have shown the potential utility of the species in plant breeding programs, we assembled a collection of bittersweet germplasm from natural populations found in Europe. This collection was analysed with conserved DNA-derived polymorphism (CDDP) and intron-targeting (IT) markers to assess genetic diversity found within and among the populations. We found that there is limited genetic variability within the collected *S. dulcamara* accessions, with a greater proportion of allelic variation distributed among populations and considerably greater population structure at higher regional levels. Although bittersweet is an outcrossing species, its population structure might be affected by its perennial self-compatible nature, reducing genetic diversity within regional populations and enhancing inbreeding leading to high interpopulation or spatial differentiation. We found that populations have been separated by local selection of alleles, resulting in regional differentiation. This has been accompanied by concurrent loss of genetic diversity within populations, although this process has not affected species-level genetic diversity. Germplasm collecting strategies should be aimed at preserving overall genetic diversity in bittersweet nightshade by expanding sampling to southern Europe and to smaller regional geographic levels in northern and central Europe.

Introduction

Bittersweet (*Solanum dulcamara* L.) is a common native European weed found across the continent. This diploid species is also found in North America, where it can be considered as an invasive species that is becoming naturalised. It serves as an alternative host for many agriculturally important diseases including the potato quarantine pathogen *Ralsonia solanacearum* (Smith) Yabuuchi *et al.*, causing bacterial wilt, and *Phytophthora infestans* (Mont.) de Bary, a dangerous oomycete that is

the causal agent of late blight (Janse, 1996). However, recent studies suggest that bittersweet may not play a key role in late blight epidemiology as it does not serve as a reservoir of inocula overwintering from the previous season (Golas *et al.*, 2010a). Late blight ranks as economically the most important disease of potato and tomato. Unprotected crops in favourable weather conditions and in the presence of an inoculum source may be destroyed within 10–14 days (Lebecka, 2008). Currently, the major defense against this pathogen is the use of fungicides. However, *P. infestans* has developed

resistance to many important pesticides and breeders have become interested in the development of resistant cultivars. Therefore, it is important to find and introduce resistance genes into the currently available potato gene pool (Gorji & Polgár, 2010). To obtain such varieties, breeders in the past have focused on the introgression of dominant resistance (*R*) genes. *Solanum demissum* Lindl., a wild potato species indigenous to Mexico, has been used to create such crops, but the resistance provided has been broken by rapidly appearing virulent races of the pathogen (Gebhardt & Valkonen, 2001). This is largely due to the introduction of both mating types to the major potato-growing areas of the world, thereby enabling the pathogen to reproduce sexually and generate new possibilities for quick adaptation through recombination of traits in the sexual cycle (Smart & Fry, 2001; Vossen *et al.*, 2003). Altogether, 11 such *R* genes have been identified and mapped in the potato genome (reviewed by Gebhardt & Valkonen, 2001), but there is still a pressing need to find new and effective resources offering resistance against novel virulent races of this pathogen. It has recently been shown that bittersweet hardly suffers from late blight and carries a new resistant gene, *Rpi-dlc1*, on the distal end of the long arm of chromosome 9 (Golas *et al.*, 2010b). This finding makes *S. dulcamara* germplasm resources valuable for plant breeding. So far, only one *Phytophthora*-resistant gene has been described from another nontuber-bearing species, *Solanum caripense* (Nakitandwe *et al.*, 2007). Therefore, it is important to study the diversity of bittersweet genetic resources to facilitate potato breeding programs.

In recent years, there has been a trend away from arbitrarily amplified dominant (AAD) markers towards gene-targeted functional markers in germplasm diversity assessment. Total genomic databases [e.g. NCBI GenBank; Potato Genome Sequencing Consortium (PGSC)] have become primary sources for marker development. On the basis of the characterisation of plant genes and gene families, new methods have been developed to analyse genetic diversity based on genomic database mining. Following this, many recent studies have suggested that polymorphism in functional regions of the genome should be exploited to achieve better estimates of genetic relationships that are relevant to plant breeding or germplasm conservation (Eivazi *et al.*, 2008; Raji *et al.*, 2009). A wide variety of DNA arrays have been presented to meet these goals. In conserved DNA-derived polymorphism (CDDP), short primers are used to generate useful genetic markers across functional domains of well-characterised plant genes (Collard & Mackill, 2009). Another recently developed technique, intron-targeting (IT), is based on the fact that intron sequences are generally less conserved than exons, and

display polymorphism due to length and/or nucleotide variation in their alleles. Primers designed to anneal in conserved exons to amplify across introns can show length polymorphism in the targeted intron (Poczai *et al.*, 2010). These markers may provide valuable new tools for genetic diversity assessment of germplasm collections as well as in other fields of plant science and breeding. However, the utility of these markers has yet to be thoroughly addressed.

The objectives of our study were (a) to study genetic diversity and relationships within a newly assembled collection of *S. dulcamara* germplasm using CDDP and IT markers and (b) to show the distribution patterns of genetic variability within/among populations, as well as (c) to assess the implications for the conservation and sustainable utilisation of bittersweet genetic resources.

Materials and methods

Plant material and *in vitro* culture

Bittersweet berries and seeds were collected from natural habitats in Europe during the growing season between 2003 and 2010 (Table 1). A total of 90 accessions were used in this study. Multiple terminals from six other species of the closely and distantly related clades of the genus *Solanum* following Weese & Bohs (2007) were chosen as outgroup exemplars. These taxa were *Solanum aviculare* Forst, *Solanum symonii* Eichler (*Archaesolanum* clade); *Solanum nigrum* L., *Solanum villosum* Mill. (*Morelloid* clade); *Solanum rostratum* Dunal (*Leptostemonum* clade) and *Solanum abutiloides* (Griseb.) Bitter & Lillo (*Brevatherum* clade). The bittersweet accessions represented 17 natural populations from European habitats. Plants were grown *in vitro* to obtain fresh tissues for DNA extractions. Seeds were surface-sterilised by a brief ethanol (70%) immersion followed by surface sterilisation treatments with a 7% (v/v) aqueous solution of sodium hypochlorite for 15 min and four washes with sterile distilled water. The sterilisation step was repeated with aqueous H₂O₂ (20% v/v) and HgCl₂ (1% m/v) solutions followed by four washing steps. Finally, seeds were placed on 30-mL solid Murashige–Skoog (MS) (Murashige & Skoog, 1962) medium supplemented with MS vitamins, 0.8% agar and 3% sucrose, with five to six seeds per vessel. Cultures were grown at 23 ± 2°C, a day length of 16 h and a light intensity of 106 μmol m⁻² s⁻¹.

DNA isolation and PCR amplification

DNA was extracted from 50 mg of fresh plant tissues according to the protocol described in Poczai & Hyvönen (2011). For CDDP amplifications, six primers

Table 1 Genetic diversity of bittersweet nightshade (*Solanum dulcamara*) accessions based on conserved DNA-derived polymorphism (CDDP) and intron-targeting (IT) markers^a

Location/Province	Habitat	ID	Collection				PP (%)	n_a	n_e	I	H_E	H_T	θ^B	F_{IS}
			Year	N	PB									
Hungary														
Keszthely	Aquatic	S001	2009	5	21	14	1.14	1.08	0.07	0.05	0.41	0.05	0.50	
Kisar	Aquatic	K001	2005	6	17	11	1.11	1.09	0.07	0.05	0.44	0.13	0.49	
Lillafüred	Montane	L001	2004	5	14	9	1.09	1.07	0.04	0.06	0.46	0.09	0.49	
Mean \pm SD					17	12	1.12	1.08	0.06	0.05	0.43	0.09	0.49	
							(0.35)	(0.28)	(0.15)	(0.22)	(0.01)	(0.05)	(0.28)	
Total Hungary					21	14	1.14	1.11	0.06	0.08	0.43	0.22	0.50	
Ukraine														
Chop	Aquatic	U341	2003	5	15	10	1.10	1.07	0.03	0.05	0.46	0.09	0.50	
Vylok	Aquatic	U342	2004	5	20	13	1.13	1.10	0.05	0.08	0.43	0.14	0.50	
Hetynia	Aquatic	U343	2007	5	15	10	1.10	1.07	0.04	0.06	0.44	0.12	0.50	
Mean \pm SD					16	11	1.11	1.08	0.04	0.06	0.45	0.12	0.50	
							(0.34)	(0.25)	(0.14)	(0.20)	(0.01)	(0.05)	(0.29)	
Total Ukraine					20	13	1.13	1.09	0.05	0.08	0.43	0.20	0.49	
Romania														
Sarasău	Aquatic	R451	2005	5	15	10	1.10	1.07	0.04	0.05	0.43	0.09	0.50	
Săpânța	Aquatic	R452	2009	5	50	34	1.34	1.21	0.12	0.18	0.40	0.15	0.50	
Mean \pm SD					32	22	1.22	1.14	0.08	0.12	0.42	0.12	0.50	
							(0.47)	(0.41)	(0.22)	(0.31)	(0.007)	(0.05)	(0.28)	
Total Romania					51	35	1.35	1.30	0.16	0.22	0.45	0.38	0.50	
Poland														
Warszawa	Plain	P561	2008	5	8	5	1.05	1.03	0.02	0.03	0.40	0.24	0.50	
Łódź	Plain	P562	2006	5	10	6	1.06	1.05	0.02	0.03	0.40	0.17	0.50	
Kraków	Plain	P563	2008	6	18	12	1.12	1.10	0.05	0.07	0.45	0.11	0.49	
Mean \pm SD					12	8	1.08	1.06	0.03	0.05	0.42	0.17	0.49	
							(0.37)	(0.21)	(0.12)	(0.19)	(0.01)	(0.03)	(0.29)	
Total Poland					24	16	1.16	1.08	0.05	0.08	0.23	0.45	0.50	
Germany														
Stuttgart	Plain	G671	2010	5	18	12	1.12	1.07	0.04	0.06	0.41	0.16	0.49	
Bonn	Plain	G672	2010	7	9	6	1.06	1.04	0.02	0.03	0.33	0.35	0.50	
Rostock	Plain	G673	2010	6	12	8	1.08	1.06	0.03	0.04	0.42	0.19	0.49	
Kiel	Plain	G674	2010	4	9	6	1.06	1.04	0.02	0.03	0.41	0.20	0.49	
Mean \pm SD						8	1.08	1.05	0.03	0.04	0.39	0.23	0.49	
							(0.33)	(0.20)	(0.11)	(0.16)	(0.02)	(0.06)	(0.28)	
Total Germany					18	12	1.12	1.06	0.03	0.05	0.33	0.35	0.50	
France														
Paris	Aquatic	F891	2010	5	7	4	1.04	1.04	0.02	0.03	0.39	0.17	0.50	
\pm SD							(0.21)	(0.21)	(0.10)	(0.14)	(0.05)	(0.11)	(0.29)	
Finland														
Helsinki	Plain	F199	2010	6	3	2	1.02	1.01	0.01	0.01	0.36	0.34	0.52	
\pm SD							(0.14)	(0.13)	(0.06)	(0.09)	(0.08)	(0.18)	(0.28)	
Mean within all populations					17	17	12	0.87	1.10	0.05	0.08	0.41	0.17	0.50
Among all populations \pm SD					90	130	90	1.90	1.64	0.49	0.33	0.38	0.76	0.50
							(0.29)	(0.37)	(0.17)	(0.23)	(0.001)	(0.007)	(0.28)	

N , the number of samples used from the population; n_a , the observed number of alleles; n_e , the effective number of alleles; PB, the number of polymorphic bands; PP (%), the percentage of polymorphism.

^aGenetic diversity indices calculated under Hardy–Weinberg equilibrium are the Shannon's information index (I) of phenotypic diversity; expected heterozygosity (H_E) or Nei's gene diversity. Indices based on Bayesian estimates not requiring Hardy–Weinberg proportions based on an f -free model (detailed settings are discussed in text): panmictic heterozygosity or total heterozygosity (H_T); fixation index (θ^B), the Bayesian analogue of F_{ST} ; estimate of inbreeding (F_{IS}).

and three primer pairs (Collard & Mackill, 2009) were used, while for the IT markers 12 primers were tested from the set described by Poczai *et al.* (2010). In the CDDP reactions, we also tried combining the primers to

amplify polymorphic regions representing DNA stretches between two identical or very similar conserved primer-binding sites. The utility of primer pairs in the case of gene-targeted marker systems was presented by

Table 2 Details about conserved DNA-derived polymorphism (CDDP) and intron-targeting (IT) primers used to assess genetic diversity in bittersweet (*Solanum dulcamara*)

Primer Name	(Putative) Gene Function	Primer Sequence (5' to 3')	Length	GC (%)	T_a	Scored Bands
CDDP primers						
WRKY-A*	Transcription factor for developmental and physiological roles	F: TGGCGSAAGTACGGCCAG	18	65	50	10
		R: GTGGTTGTGCTTGCC	15	60		
WRKY-B	Implicated in secondary metabolism, abiotic and biotic stress and cellular morphogenesis	F/R: GCCCTCGTASGTSGT	15	67	50	12
MYB		F/R: GGCAAGGGCTGCCGC	15	80	57	9
ERF*	Transcription factor involved in plant disease-resistance pathway	F: CACTACC GCGSCTSCG	17	77	51	12
		R: GCSGAGATCCGSGACCC	17	77		
KNOX*	Homeobox genes that function as transcription factors with unique homeodomain	F: AAGGGSAAAGCTSCCSAAG	18	44	48	10
		R: CACTGGTGGGAGCTSCAC	18	61		
MADS-A	Involved in controlling floral organ initiation and development	F/R: ATGGGCCGSGGCAAGGTGC	19	68	57	15
MADS-B		F/R: CTSTGCGACCGSGAGGTC	18	72	53	12
ABP1-2	Auxin-binding protein	F/R: ACSCCSATCCACCGC	15	60	50	9
ABP1-3		F/R: CACGAGGACCTSCAGG	16	69	50	10
IT primers						
Adk-242	Adenylate kinase gene	F: TGCTTTAAAGTCGCACCA	19	42	55	4
		R: TTATATCCGGAGCATGTCCAC	21	47		
Adk-795	Adenylate kinase gene	F: GCATGGTCTTTCCTTCCTG	20	50	54	4
		R: TGGGCCAGGAATTTGCTATC	21	47		
Cat-232	Potato catalase gene	F: AGGAGGCGGATCTAGCCTTA	20	55	55	2
		R: TGCAAGAAAAGGGGTGTCGT	20	50		
Cat-260	Potato catalase gene	F: TGACAACAAATGCTGGTGGT	20	45	53	3
		R: AAGGTGGCAAGCTTCTCAAT	20	45		
GPSS-275	ADP-glucose pyrophosphorylase small subunit gene	F: CTTTGTATGGGGCAGATTA	20	45	53	3
		R: CAGCTTCCTGTGATCATCAG	20	55		
GPSS-943	ADP-glucose pyrophosphorylase small subunit gene	F: TCATTGGTGAAGTTGTGTGA	21	43	53	7
		R: ACCACGGAATGGTGAATCTT	20	45		
INHWI-509	Wound-inducible proteinase inhibitor I gene	F: TGAACTCTCTTGGCAGAA	20	45	54	2
		R: TTCTGGCCACCTTTGTTTTTC	20	45		
INHWI-545	Wound-inducible proteinase inhibitor I gene	F: TCAAGTTTGTCCATCTTGT	21	38	54	4
		R: TCGTGCCAAGAGAGTTTCAA	20	45		
InG-220	Invertase gene	F: ACAGGAATCACACCTGCACA	20	50	54	3
		R: TCTGCACCCTTAAGTCCACA	20	50		
		R: TCTGCACCCTTAAGTCCACA	20	50		
LBr-G9	Transducin family protein	F: TGGATCTGAAGATGGCACTG	20	50	55	3
		R: TTGCTCTCAAATCCCACACA	20	45		
S2-317	Self-incompatibility locus linked	F: CGGCCAGTTACAATTCTGC	19	52	55	6
		R: AATCCAGTGGTGGTCCAGAG	20	55		
Poni1a-718	Potato membrane protein	F: GGTGGTGGTGGTACTCAGT	20	60	55	3
		R: CCCARRGGCATTAACTCC	20	50		

GC (%), Guanine-Cytosine content (%); ADP, Adenosine diphosphate.

*Primers used in combination. Code for mixed base S = G or C.

Gorji *et al.* (2011). For further details on the primers, see Table 2. PCRs were performed in 10 μ L volumes containing 5 μ L nuclease-free water, approximately 20 ng template DNA, 0.5 μ M of each primer, 0.2 mM

deoxyribonucleotide (dNTP) (Fermentas, Vilnius, Lithuania), 1 μ L 10 \times PCR buffer (1 mM Tris-HCl, pH 8.8 at 25 $^{\circ}$ C, 1.5 mM MgCl₂, 50 mM KCl and 0.1% Triton-X-100) and 0.5 U of DyNazyme II (Finnzymes, Espoo,

Finland) polymerase. All reactions were performed with the following settings in a MasterCycler ep96 (Eppendorf, Hamburg, Germany): 2 min at 94°C for initial denaturation, 35 cycles of 30 s denaturation at 94°C, 1-min annealing at optimal temperature and 2-min extension at 72°C, followed by a final extension for 5 min at 72°C. Amplification products were separated on 1.5% agarose gel (GE Healthcare, Little Chalfont, UK) in 0.5× Tris/Borate/EDTA (TBE) buffer (220 V, 0.5 h) and stained with ethidium bromide. Each amplification contained one negative control and one positive control, to check the reliability of the primers and the patterns produced.

Band scoring protocol

The resulting gels transformed to electropherograms were scored for polymorphic peaks as absent/present (0/1) for both marker systems (Fig. 1). We assumed that fragments of equal length had been amplified from homologous loci representing a single, dominant locus with two possible alleles. This assumption might be challenged by further investigations into the nature of both CDDP and IT markers. Only well-resolved, distinct bands were coded in the data matrix. The presence of a determined locus was confirmed using the program Phoretix 1D Pro (TotalLab, Newcastle, UK) by verifying the peaks of bands produced during gel analysis. The scoring protocol of Holland *et al.* (2008) was applied to prevent problems associated

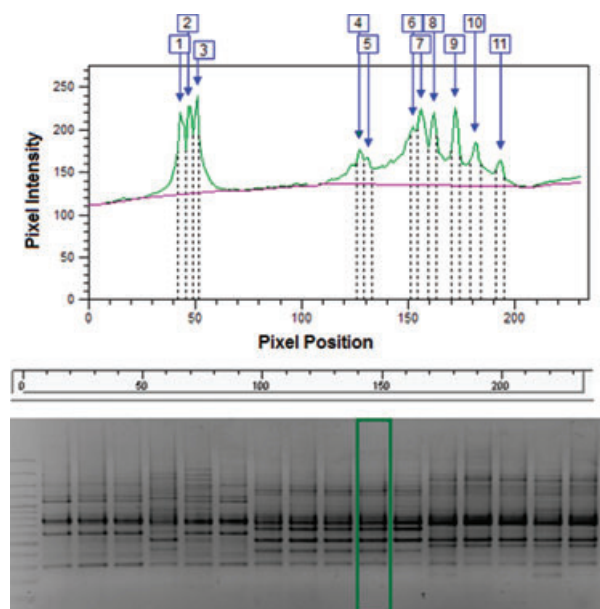


Figure 1 Example amplification patterns in *Solanum dulcamara* with conserved DNA-derived polymorphism (CDDP) primers WRKY-A. Above gel profile, an example transformation of banding patterns to an electropherogram is represented using Gaussian filtering.

with multiple banding patterns. This conservative scoring minimises problems associated with uneven amplification among samples and poor amplifications of larger fragments for degraded DNA samples. Only fragments that showed even amplification in all samples were scored (no fragments, where any sample exhibited poor amplification, less than 20 pixels with Gaussian filtering, were used), and evaluation was halted at the size (usually 100–50 bp) where poorer quality samples began to lose monomorphic peaks in our data.

Genetic diversity analysis

To determine the level of genetic variability and genetic differentiation within and among populations of *S. dulcamara*, the number of polymorphic bands (PBs) and the percentage of polymorphic bands (PPs) were calculated for the united CDDP and IT dataset. A locus was considered polymorphic when the band was present at a frequency between 5% and 95%. To further characterise the levels of genetic diversity, the observed number of alleles (n_a) and the effective number of alleles (n_e) were calculated according to Kimura & Crow (1964) for each population. The n_e was defined as the inverse of the homozygosity, which is a lower bound for the actual number of alleles in the population. We also calculated Shannon's information index (I) of phenotypic diversity (Lewontin, 1972) across all loci. Further expected heterozygosity (H_E) or Nei's gene diversity (Nei, 1973) was calculated from allele frequencies based on the square root of the frequency of the null (recessive) allele. All calculations were performed within and among different populations of *S. dulcamara* using the program POPGENE v1.32 (Yeh *et al.*, 1997).

Bayesian estimate of population divergence

Given that the calculation of allele frequencies based on dominant markers requires the assumption of Hardy–Weinberg equilibrium, a more direct estimate of the relative magnitude of population divergence in the combined dataset was obtained using a Bayesian approach developed by Holsinger (1999). This method does not assume that genotypes are in Hardy–Weinberg proportion within populations and takes full advantage of the information provided by molecular markers (Holsinger & Wallace, 2004). The program HICKORY v.1.0.4 (Holsinger & Lewis, 2003) was used for all calculations through several runs with default sampling parameters (burn-in = 50 000; sample = 250 000; thin = 50) to ensure that results were consistent. With this sampling strategy, every 50th sample was retained for *posterior* calculations. The *posteriori* distribution of θ^B (analogous to Wright's F_{st} ; θ^I option in HICKORY)

was approximated through a Markov Chain Monte Carlo (MCMC) simulation. This method also provides some information about the degree of within-population inbreeding by calculating f (estimate of F_{IS}). We also calculated the panmictic heterozygosity, or total heterozygosity (H_T), based on mean allele frequencies during the same MCMC runs. The Bayesian estimates of genetic diversity were calculated under four models: (a) *full* model (with noninformative *priors* for f and θ^B); (b) $f = 0$ (assuming no inbreeding); (c) $\theta^B = 0$ (assuming no population structure) and (d) f -free (allowing the incorporation of uncertainty about f into the analysis). The model choice was based on the deviance information criterion (DIC), which combines a measure of model fit ($Dbar$) with the measure of model complexity (pD). The preferred model is the one with lowest DIC value representing the best compromise between model fit and number of parameters.

Phylogenetic analysis

To examine phylogenetic structure within and among populations, polymorphic loci from both marker systems were scored as binary characters for parsimony analysis to produce a matrix of 143 informative characters. Searches for most parsimonious trees were conducted using PAUP* v.4.0b10 (Swofford, 2002) with stepwise random taxon addition and TBR branch swapping. Branches were collapsed when the minimum length was zero and all character transitions were equally weighted. An initial search of 50 000 replications was performed saving only one tree of length ≥ 1 on each replication (nchuck = 1, chuckscore = 1). Subsequently, a second search was performed with no limits on the number of trees saved (nchuck = 0), starting with the trees held in memory from the first step. Trees were rooted with *S. abutiloides* and *S. rostratum* based on the results of Weese & Bohs (2007). Nonparametric bootstrap analyses were conducted on each dataset with 1000 replications of two full heuristic searches each, saving a maximum of 500 trees on each replication (nchuck = 500, chuckscore = 1).

Results

Genetic diversity and estimated levels of inbreeding

Diversity estimates were based on simultaneous analysis of the total combined dataset, containing 90 terminals and 143 characters representing 17 *S. dulcamara* populations from seven regions. One hundred and thirty loci (90%) were polymorphic among the total population. The number of PBs varied between 3 and 50 with the lowest value (2%) detected in the Finnish Helsinki population and the highest (34%) occurring in the Romanian Săpânța

population. Population genetic parameters, namely, the observed number of alleles, the number of effective alleles, Shannon's information index, expected and observed heterozygosity, as well as Bayesian estimates of total heterozygosity, inbreeding and fixation index are shown in Table 1 for all populations. All these calculated indices of genetic diversity confirm that variability is distributed among populations, as all values were slightly higher than those calculated for each population or region. Within populations, the effective number of alleles (n_e) varied between 1.01 and 1.21, whereas the observed number of alleles (n_a) ranged between 1.02 and 1.34. The values of Shannon's information index (I) were similar to expected heterozygosity values, ranging from 0.01 to 0.12, while expected heterozygosity (H_E) averaged across all bitter-sweet populations ranged from 0.01 to 0.18 with an average value of 0.08. The Bayesian estimate of fixation index (θ^B) was slightly higher compared to heterozygosity calculations assuming Hardy-Weinberg proportions, ranging from 0.05 to 0.35 with an average value of 0.17. The estimate of total heterozygosity with the Bayesian approach (not assuming Hardy-Weinberg proportions) returned posterior distributions ranging from 0.33 to 0.46 with an average of 0.41. These estimates for each population differ from those obtained under Hardy-Weinberg equilibrium, while the Bayesian estimates for among the whole population matched those obtained under the above-mentioned criteria. Inbreeding estimates were more similar within and among populations, ranging from 0.49 to 0.52 with an average of 0.50.

In the Bayesian analysis of population structure using the full and $f = 0$ models, the posterior mean estimates of θ^B were almost equal ($\theta^B = 0.74 \pm 0.008$ and 0.73 ± 0.008 , respectively). The f -free model resulted in slightly higher values ($\theta^B = 0.76 \pm 0.007$). All models showed closely similar estimates for the Bayesian fixation index (θ^B), irrespective of different parameters and settings. Of these four models, the f -free model was the best fitting, having the lowest DIC value of 1968.37. On the basis of these results, the f -free model was preferred over the others. With this choice we incorporated all uncertainty in the estimations of f during the MCMC run. Fig. 2 shows the results derived from the f -free run with default settings, illustrating the accurate and precise estimates of θ^B , while the estimates of f , not surprisingly, are uninformative. Panels (c) and (d) show that the sampler thoroughly explored the relevant parts of parameter space.

Parsimony analysis

The parsimony analysis resulted in 188 940 equally parsimonious trees (EPTs) of length 570. The consistency index (CI = 0.25) indicates significant homoplasy, an

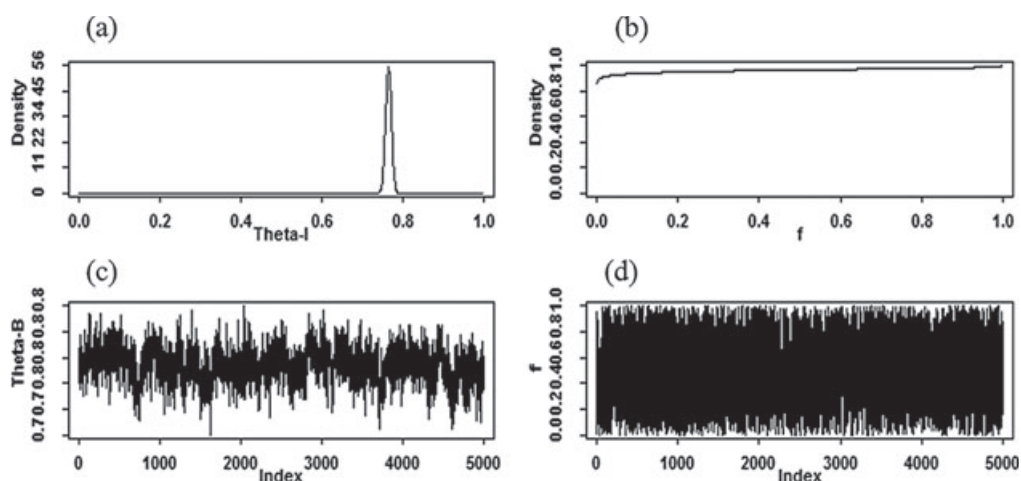


Figure 2 Convergence of the sampler and posterior densities for θ^B and f with the dataset generated by conserved DNA-derived polymorphism (CDDP) and intron-targeting (IT) markers. Estimates are based on an f -free model. (a) Posterior density of θ^B . (b) Posterior density of f . (c) Sample history of θ^B . (d) Sample history of f .

average of four state changes per character. Fig. 3 shows the strict consensus tree with bootstrap support values and a representative phylogram. High bootstrap values for many of the deeper nodes and resolution in the strict consensus indicate significant phylogenetic structure at the level of regional population groups, while at lower interpopulation levels and within populations, structure is mostly ambiguous or weakly supported. Well-supported relationships are strongly geographically patterned, with the Finnish specimens forming a clade that is sister to the remainder of the samples. Within this latter group, the Western European specimens are sister to a clade of Eastern European exemplars. Clades of German, French and Ukrainian specimens also show high support values. The Polish specimens are effectively unresolved within the Eastern European clade, while the Romanian exemplars occur in two distinct groups. One of these, consisting of specimens from the Sarasău population together with a single representative of the Săpânța population, forms a clade that is sister to a combined group of Ukrainian, Hungarian and other Romanian exemplars, while in the other group the remaining Romanian Săpânța population specimens together with the Hungarian exemplars.

Discussion

Utility of CDDP and IT markers

The results confirm that both CDDP and IT markers are suitable for characterising bittersweet genetic resources and that they can possibly also be used in other solanaceous plants, alone or in combination. The successful transferability and cross-species amplification

of IT markers may result from the relative conservation of exon–intron junctions and gene structures across solanaceous plants. This high degree of conservation may be due to *Solanaceae* genomes having undergone relatively few genomic rearrangements and duplications and therefore having a similar gene content and order (Mueller *et al.*, 2005), making it easy to use the same IT primers across diverse *Solanum* species. This phenomenon is valuable for generating functional markers directly related to gene regions and facilitating the discovery of specific markers linked to valuable traits (e.g. resistance genes) in bittersweet germplasm. It is also possible to tag resistance mechanism-related genes with the IT system to provide new opportunities for utilisation of bittersweet genetic resources. This is because both CDDP and IT use primers based on allele sequences of functionally characterised genes, and thus specific banding patterns corresponding to plant phenotypes can be identified. In a previous study using IT markers, Cernák *et al.* (2008) showed that a portion of the catalase gene is linked to Potato virus Y (PVY) resistance and that this gene might play a role in extreme defense mechanisms in solanaceous plants. However, the development of such markers depends on the availability of robust genomic databases. In the case of *Solanum*, the comprehensive databases of the PGSC (Visser *et al.*, 2009) provide the ultimate source for further marker development. Such databases hold several target genes for both IT and CDDP development. Functional gene characterisation criteria might be limiting factors as not all the genes would be sufficient to establish gene functions in an agronomic sense. The crucial question is whether useful allelic variation can be identified for all genes of ecological and agronomic relevance in crops and germplasm collections.

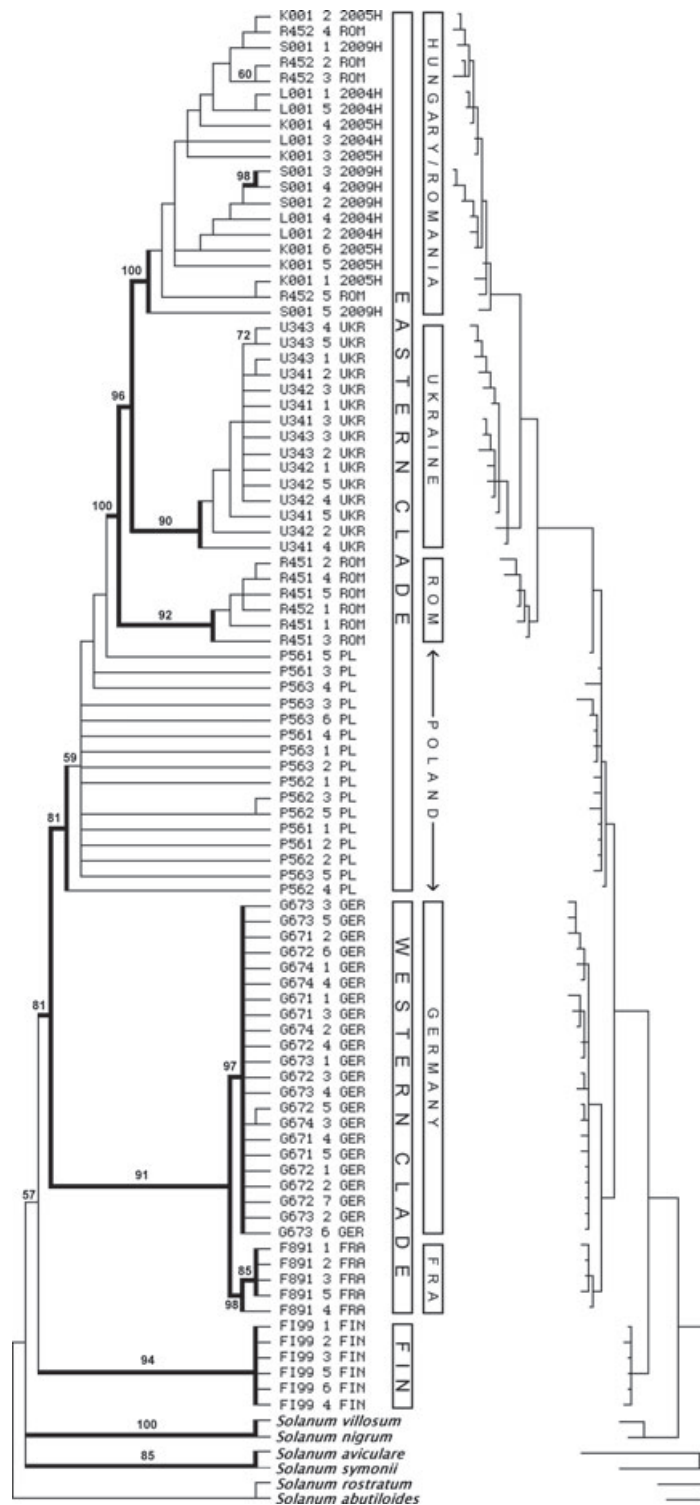


Figure 3 Strict consensus (left) of 188 940 equally parsimonious trees found in parsimony analysis of combined conserved DNA-derived polymorphism (CDDP) and intron-targeting (IT) marker data [consistency index (CI) = 0.25, rescaled consistency (RC) = 0.22, retention index (RI) = 0.89]. Numbers above branches are bootstrap support values and branches supported at $\geq 80\%$ are in bold. A representative phylogram is on the right. Ingroup terminals (*Solanum dulcamara*) are indicated by population code (Table 1), specimen number and country code. Vertical boxes signify major regional (left) and smaller geographical (right) clades. Arrows indicate nonmonophyletic assemblages.

Genetic diversity in *Solanum dulcamara*: exploring potential genetic resources

Population genetic tools have not been extensively used to study microevolutionary processes such as population subdivision and structure and genetic drift in *Solanum*. However, numerous studies have measured genetic variability at the macroevolutional scale within and among species or species complexes (Jiménez *et al.*, 2008; Manoko *et al.*, 2008; Fu *et al.*, 2009). In this regard, our knowledge of the differentiation and population genetics of *S. dulcamara* is limited. However, there have been recent attempts to study the genetic diversity of this European species (Golas *et al.*, 2010c). The lack of detailed knowledge about *S. dulcamara* might be due to the fact that plant breeding-directed genetic resource exploration in *Solanum* has mainly been undertaken in Central and South America. The potential utility of European species such as *S. dulcamara* or *S. nigrum* as sources of valuable traits (mainly resistance genes) has not been thoroughly studied. Plant breeding has benefited from previous studies aimed at exploring species boundaries and genetic diversity in South America (Ames & Spooner, 2010; Rodríguez *et al.*, 2010). These genetic diversity studies of wild *Solanum* species used various marker systems (Albrecht *et al.*, 2010; Li *et al.*, 2010). The consensus finding was that self-incompatible species of *Solanum* are more diverse than self-compatible (SC) taxa (Albrecht *et al.*, 2010). The *a priori* assumption of the outcrossing nature of bittersweet was consistent with prior expectations based on all calculated genetic diversity values in our analysis. The mating system of a species has a significant effect on the distribution of genetic variation. Bittersweet, on average, has a greater proportion of allelic variation distributed among populations than within each local population studied, this being consistent with the considerably greater phylogenetic structure found at higher levels in the phylogenetic analysis. These findings are congruent with those obtained by Golas *et al.* (2010c) and fit well with previous expectations about SC *Solanum* taxa. In *Solanum*, self-incompatibility (SI) seems to be the ancestral state, and evolutionary transitions to the SC state are irreversible (Ilgic *et al.*, 2004). *S. dulcamara* is an outcrossing, but SC perennial species (Vallejo-Marín & O'Brien, 2007), explaining the low within-population genetic diversity detected in our study. It has been shown for other species that self-compatibility is strongly associated with changes in mating system and genetic diversity (Mable *et al.*, 2005). However, the extent to which the breakdown of an SI state increases self-fertilisation in natural populations of *Solanum* is not known (Vallejo-Marín & O'Brien, 2007). Our results show that *S. dulcamara* is a species

with a variety of narrowly adapted genotypes with low within-population genetic diversity but high phenotypic plasticity, leading to greater population success in a wide variety of habitats from mountain ranges to plains and even wetlands. Recent studies of the breeding system of bittersweet by Golas *et al.* (2010c) found only a few individuals that could be the result of self-fertilisation when cross-pollen was available. A similar situation was found in *Phlox drummondii* Hook. (Levin, 1996) when alien pollen containing a genetic marker was mixed with an equal quantity of normal pollen and about 85% of the progeny were shown to be produced by cross-fertilisation. This could indicate that while bittersweet prefers cross-pollination, self-fertilisation might serve as insurance policy; if nothing better comes along it provides the ability to produce seeds. This trait could be a competitive advantage under unfavourable environmental conditions, thereby broadening the adaptive capability of the species.

Population structure and divergence in *Solanum dulcamara*

Population structure is determined by many biological and ecological factors such as breeding system, genetic drift, and mutation rate as well as habitat fragmentation. Populations of *S. dulcamara* in this study had high genetic structure as populations in different locations were genetically different, resulting in local or regional fragmentation. In the parsimony analyses, high bootstrap values for deeper nodes suggest that at inter-regional levels in particular, population groups are moderately to highly isolated, with nested hierarchical (phylogenetic) relationships predominating. The high support values for well-resolved regional groupings were obtained despite abundant homoplasy in the data (CI = 0.25), indicating that while most individual alleles have been gained and lost on multiple occasions and/or convergently, these patterns are more or less congruent across different characters and thus likely to reflect shared ancestry. This is consistent with the high retention index (RI = 0.89; Farris, 1989), which indicates that most of the observed character changes are synapomorphic. In other words, the gain and loss of alleles is rather plastic but nonetheless phylogenetically informative. Field observations of individual populations found that there were numerous seedlings close to their presumed maternal plants, with only a few more distant propagules. The population structure of bittersweet might be affected by the proximity of parents and their progeny. A good indicator of this could be the high level of inbreeding detected in all populations. However, Bayesian inbreeding (f or Bayesian F_{IS}) estimates made here

should be interpreted cautiously according to Holsinger *et al.* (2002). Inbreeding could also be the cause of differences observed between diversity estimates based on allelic frequencies (H_E , I) and Bayesian estimates of total heterozygosity (H_T). These differences indicate that population level processes might not be perfectly depicted under the assumption of Hardy–Weinberg equilibrium. In perennial plants, progeny might stay close to parent plants, increasing the probability that crosses between neighbours will tend to involve relatives. This could disrupt random mating, as perennial parents could contribute to each generation year after year, increasing the level of inbreeding. The longevity of these plants makes crossings between generations possible, making genotypes more related than the average within a population as presented by Hamrick & Godt (1996). This feature of bittersweet leading to fragmented distributions with low connectivity could further limit gene flow between populations and enhance inbreeding with the effect of genetic drift, again leading to high interpopulation differentiation. The differentiation among populations could have arisen by genetic drift or even by inbreeding over a very long period, as the populations were separated inducing local selection of alleles. The greater differentiation of populations comprising the Eastern and Western clades could be due to concurrent loss of genetic diversity within populations because of loss of habitats containing genetically unique populations. However, although different populations may lose different alleles, a large number of alleles may nonetheless be maintained across the species as a whole (Spencer *et al.*, 2000). This is a possible reason why genetic drift does not appear to have resulted in decreased genetic diversity at the species level in bittersweet, while this mechanism can still increase population differentiation. The moderate levels of variation observed among all populations, together with the relatively common occurrence of bittersweet in Europe, suggest that drift – if it happened – has not played a strong role recently. However, it could have led to differentiation in the past, without this being apparent. Postglacial expansion from different southern European refugia is an obvious historical process that could have resulted in the strong inter-regional phylogenetic structure we observed in our predominantly northern and central European collections. Focused sampling in Mediterranean regions and in the Iberian Peninsula might in this case be expected to uncover greater genetic diversity as well as helping to reveal the origins of northern European regional differentiation. The data presented here do not allow us to make further inferences on the historical processes, which might have caused a substantial loss of genetic diversity and formed a genetic bottleneck. An epidemic

disease or habitat loss could have been responsible for these patterns or alternatively it is possible that gene flow occurs throughout the entire European population making the species uniform. However, the latter would require that the European bittersweet population would be better regarded as a larger metapopulation, consisting of spatially separated smaller populations that interact at some level.

Bittersweet germplasm management

The effective management of bittersweet germplasm could provide new resources for resistance breeding in potato. Renewed interest in *R*-gene-based resistance against late blight has resulted in the identification of numerous wild *Solanum* species as potentially valuable sources of resistance, bittersweet being a prime example (Golas *et al.*, 2010*b*). For this reason, we characterised accessions for *ex situ* germplasm management in order to eliminate mislabelled redundancies and to obtain complete knowledge about the variability preserved in the collections. We found that there is limited genetic variability within the collected *S. dulcamara* accessions. The level of variability is similar to that found by Golas *et al.* (2010*c*) in accessions collected over a different geographic area, with many of them from the Netherlands. Germplasm collecting strategies should be aimed at capturing the largest amount of genetic diversity at the lowest possible cost, while aiming to preserve unique allele combinations. This can be achieved by sampling as many populations as possible from a wide variety of locations and regions, thereby providing insights into the partitioning of genetic diversity, as genetic diversity is preserved among populations at the species level. The observed differences between the analysed European accessions suggest that members of the Eastern and Western European clades are characterised by different alleles. Because strong spatial structure was detected in bittersweet at the species level, management should be aimed at preserving overall genetic diversity in germplasm collections. Sampling more sites and the use of different markers – possibly co-dominant ones – would be necessary to test for historical evolutionary forces that might have shaped the population structure. This should be incorporated into collection programs by separate sampling of regions, including other European areas not sampled for this study. As well as possibly expanding sampling to southern Europe. The phylogenetic structure showed in our data at smaller regional geographic levels suggests that denser sampling in northern and central regions might also uncover considerably more genetic diversity. According to Ellstrand & Elam (1993), when most of the genetic

diversity is portioned among populations rather than within them, a germplasm management and collection plan should include a large number of populations. The most diverse populations found in Romania could be good sources for obtaining representatives of the Central European region. Special attention should be given to populations growing alongside waterways, as transmission of seeds by rivers and streams contributes to the exchange of alleles between different populations even across large regions. This might explain why members of one Romanian population (Săpânța) are nested within a relatively closely located Hungarian group. Our own observations suggest that birds drinking from rivers and streams often feed on the attractive red berries of bittersweet and thus may play an important role in seed distribution throughout these habitats. Bird dispersal was also found to be very important for other solanaceous species with red or orange fruits (Fukuda *et al.*, 2001; Poczai *et al.*, 2011). Dispersal both by water and frugivorous birds may play an important role in promoting allele exchange between riverside populations.

Concluding remarks and future directions

Our results could be used to specifically identify accessions or groups to develop efficient germplasm management strategies. To further utilise the assembled collections, careful planning regarding optimal strategies is required. The moderate genetic diversity among *S. dulcamara* populations (0.33) showed by CDDP and IT markers indicates the potential for further exploration and collection of specimens over a wider part of the global distribution range for the purpose of genetic improvement of solanaceous crop gene pools. In the *Solanaceae*, crops such as potato, tomato and pepper have greatly benefited from the use of wild relatives in breeding programs (Albrecht *et al.*, 2010). Virtually all the disease-resistance genes introduced in modern tomato varieties originated in related wild species (Rick & Chetelat, 1995). Up till now, these traits have mostly been derived from the South and Central American *Solanum* gene pool. Bittersweet nightshade provides an opportunity to explore the potential of Eurasian/European species of *Solanum* as sources of genetic resources to further stimulate plant breeding programs. To properly assess the usefulness of bittersweet for practical breeding programs, further studies are required to investigate the causes of the differentiation between the two major clades identified. These studies would also either corroborate or require us to modify our estimations of high levels of inbreeding within populations and might provide new insights into the biology, breeding system and history of this species

in its native range, as well as in habitats where it is considered to be introduced.

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