

# Genetic structure of European accessions of *Solanum dulcamara* L. (Solanaceae)

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**Abstract** *Solanum dulcamara* (bittersweet) is one of the few native species of *Solanum* present in Europe. It is a common weed that occupies a wide range of habitats and is often found in the direct vicinity of cultivated potatoes (*Solanum tuberosum*), where it could transmit diseases. A broad sampling of European *S. dulcamara* accessions was carried out to gain insight into the population structure and crossing preferences of this species. Three amplified fragment length polymorphism (AFLP<sup>®</sup>) primer combinations generating 288 polymorphic fragments were used to analyze 79 bittersweet accessions (245 individuals). Dendrograms

revealed a low level of genetic polymorphism in the bittersweet populations, caused partially by the out-crossing nature of this species.

**Keywords** *Solanum dulcamara* (bittersweet) · Section *Dulcamara* · AFLP · Genetic distances

## Introduction

*Solanum dulcamara* L., commonly named bittersweet or climbing nightshade, is one of the few native species of *Solanum* L. in Europe. It is a well-known host for the potato quarantine pathogen *Pseudomonas solanacearum* (Smith) Smith, a causal agent of bacterial wilt or brown rot (Olsson 1976; Elphinstone et al. 1996; Janse 1996), and may play an important role in potato late blight epidemiology (Flier et al. 2003; Dandurand et al. 2006). In some countries in North-West Europe where potato is cultivated, *S. dulcamara* was subjected to eradication programs from the natural vegetation, aimed at preventing the spread of *P. solanacearum*.

*Solanum dulcamara* is one of the about 1,500 species of the genus *Solanum* (Weese and Bohs 2007), which has a cosmopolitan distribution (D'Arcy 1991). *Solanum dulcamara* belongs to section *Dulcamara* (Moench) Dumort. of subgenus *Potatoe* (G. Don) D'Arcy (Child and Lester 2001). Subgenus *Potatoe* also includes the cultivated potato and its wild relatives. Section *Dulcamara* has been studied by several authors, and there is no consensus on the size and content of the section.

Child and Lester (2001) recognized three sections in the Dulcamaroid taxa: section *Jasminosolanum* Bitter ex Seithe, section *Californisolanum* A. Child, and section *Dulcamara*, the latter containing four to five Eurasian

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species. Recent analyses of the *ndhF*, *trnT-F*, and *waxy* genes (Bohs 2005; Weese and Bohs 2007) showed that the Dulcamaroid clade, including *S. dulcamara*, clusters closely to a Morelloid clade (containing the species of *Solanum* section *Solanum*).

*Solanum dulcamara* is a species widely naturalized in the entire Holarctic area (Horvath et al. 1977). This diploid ( $2n = 24$ ) perennial plant occurs in contrasting environments, from wet habitats of irrigation ditches, river banks, and lake shores to dry areas of dunes and plains. Seeds are mainly dispersed by birds. Fruits as well as vegetative parts of the plant can be transported by water.

The present study was conceived to gain information on the infraspecific variation within *S. dulcamara*. We used a collection covering a broad range of variation in Europe, both with respect to geographical provenance and to different habitats. AFLP data were generated to assess the genetic distances among the populations. Furthermore, to better understand the mechanism of gene flow between *S. dulcamara* populations, we established the breeding system of this species in its natural environment, also using AFLP data.

## Materials and methods

### Plant material

Seeds of *S. dulcamara* from the Solanaceae collection of the Radboud University Botanical and Experimental Garden, Nijmegen (The Netherlands) were used. In addition we used seeds and cuttings collected in 2005 and 2006 from *S. dulcamara* plants in their natural habitats.

The accession numbers are abbreviated, e.g.: accession number 934750233 is abbreviated to 93233 or A54750207 to A5207. Passport data concerning the accessions used can be found at <http://www.ru.nl/bgard/>. All plants were cultivated at the Radboud University Botanical and Experimental Garden. Plants were grown in the greenhouse under long-day conditions (16 h day/8 h night). Supplementary light was given by using high-pressure sodium lamps (SON-T 600 W). Plants were grown in pots filled with a standard soil mixture (Lentse Potgrond no. 4). Regularly, the plants were fertilized with Kristallon Blauw (Yara Benelux B.V. Vlaardingen).

### Genomic DNA isolation

Total genomic DNA was isolated from young leaves using the Wizard genomic DNA purification kit (Promega), according to the protocol supplied by the manufacturer. A pestle was used to grind 40 mg fresh plant material in liquid nitrogen to fine powder. The concentration of DNA

was assessed using a spectrophotometer (Pharmacia Biotech: GeneQuant II), and the quality of the DNA was checked by electrophoresis in a 1% agarose gel stained with ethidium bromide.

### Genomic profiling

The AFLP method was used to estimate genetic distances among *S. dulcamara* accessions. AFLP was performed according to Vos et al. (1995) with minor modifications. DNA (0.5  $\mu$ g) was digested with *EcoRI* and *MseI* restriction enzymes (Fermentas, Germany). After ligation and pre-amplification, selective amplification was performed using D4 dye (Beckman Coulter) with labeled *EcoRI* primer together with unlabeled *MseI* primer using a GeneAmp9600 thermocycler (Perkin Elmer, USA). Three primer combinations were used, each with three selective nucleotides: *EcoRI* + AAC/*MseI* + CAC, *EcoRI* + AAC/*MseI* + CAT, and *EcoRI* + ACC/*MseI* + CAT. Polymerase chain reaction (PCR) products after selective amplification were diluted ten times in sample loading solution (SLS; Beckman Coulter). Two microliters of this dilution were added to 38  $\mu$ l SLS containing 0.2  $\mu$ l CEQ DNA size standard 600 (Beckman Coulter).

The fragments were analyzed using a Beckman Coulter 8000<sup>TM</sup> fragment analysis system with default values of study parameters, with the exception of size standard (600) and model of study (cubic).

To investigate the breeding system of the species occurring in natural conditions, the AFLP method was performed as described above, except that *EcoRI* primer labeled with  $\gamma$ 33P (MP Biomedicals, USA) was used for selective amplification. Two primer combinations each with three selective nucleotides (*EcoRI* + AAA/*MseI* + CCC, *EcoRI* + AAC/*MseI* + CAA) were used. Selective PCR products were separated on 5% polyacrylamide gels, dried on paper, and visualized by exposure to X-ray film (Kodak BIOMAX MR) for 48 h. The generated radiograms were scored manually, which was feasible due to the smaller number of markers.

### Genetic distances among accessions of *S. dulcamara*

Two hundred forty-five individuals (79 accessions) of *S. dulcamara* (Table 1; Fig. 1) were examined. For comparison, three accessions of *Solanum* species belonging to section *Solanum* were included (*S. americanum* Mill., *S. villosum* Mill., and *S. nigrum* L.). Combined results from three AFLP primer combinations generated the data matrix from which monomorphic and single fragments (present only in one individual) were removed, creating a dataset containing 288 characters. This dataset was analyzed by using NTSYS-pc<sup>®</sup> version 2.11T (Rohlf 2000).

The SimQual program, for qualitative data with both the simple matching (SM) and Jaccard (J) coefficients, was used to generate similarity matrices. Clustering was performed using the SAHN option and the unweighted pair-group method (UPGMA).

Estimation of the breeding system of *S. dulcamara* occurring in its natural environment

Three different mother plants were collected together with their respective mature berries in Castricum aan Zee, Egmond aan Zee, and Uitgeest (The Netherlands). Cuttings were rooted and maintained, and seeds were extracted. Eight seeds per mother genotype were sown. DNA of the three mother plants and eight offspring genotypes were compared using AFLP.

## Results

Genetic distances among accessions of *S. dulcamara*

The purpose of this experiment was to gain insight into the population structure of *S. dulcamara*. We examined the level of genetic variation within and among 79 bittersweet accessions growing in various habitats and different geographical regions, from all over Europe, with an emphasis on Dutch material (51 accessions).

The UPGMA cluster algorithm was applied to similarity matrices constructed with the SM and Jaccard coefficients, to graphically visualize genetic distances between accessions of this species. The generated dendrograms were compared with each other, and no important differences were found (only small differences occurred in the

**Table 1** Accessions used to test genetic distances among accessions of *Solanum dulcamara*

Map no.	Accession code	Habitat	Individuals used	Map no.	Accession code	Habitat	Individuals used	Map no.	Accession code	Habitat	Individuals used
1	93233	Aquatic	4	28	A5249	Aquatic	3	54	A5234	Aquatic	4
2	A5005	Aquatic	4	29	A5250	Aquatic	2	55	A5235	Aquatic	2
3	A5007	Aquatic	4	30	A5251	Dunes	3	56	A5236	Dry	3
4	A5008	Aquatic	4	31	A5253	Dunes	4	57	A5237	Unknown	3
5	A5009	Aquatic	2	32	A5254	Dunes	4	58	A5238	Unknown	4
6	A5101	Aquatic	1	33	85003	Unknown	3	59	A5239	Unknown	4
7	A5102	Aquatic	1	34	88034	Unknown	3	60	A5242	Aquatic	4
8	A5170	Aquatic	1	35	88057	Unknown	2	61	94001	Unknown	3
9	A5171	Dry	1	36	91008	Unknown	4	62	A4147	Unknown	4
10	A5172	Plains	1	37	91046	Unknown	4	63	A4148	Unknown	4
11	A5173	Plains	4	38	91081	Unknown	3	64	A4149	Unknown	4
12	A5174	Dunes	4	39	92023	Unknown	4	65	A4151	Unknown	4
13	A5175	Dunes	1	40	92109	Unknown	4	66	A5002	Unknown	4
14	A5176	Dunes	4	41	92195	Unknown	4	67	A5066	Aquatic	1
15	A5177	Dunes	4	42	A5191	Dry	4	68	A5067	Aquatic	4
16	A5178	Dunes	3	43	A5192	Dry	4	69	A5068	Aquatic	1
17	A5179	Dunes	4	44	A5196	Aquatic	1	70	A5069	Aquatic	4
18	A5185	Aquatic	3	45	A5197	Unknown	3	71	A5180	Dry	4
19	A5187	Aquatic	1	46	A5198	Aquatic	3	72	A5181	Aquatic	3
20	A5188	Aquatic	4	47	A5200	Aquatic	4	73	A5183	Aquatic	4
21	A5189	Aquatic	3	48	A5201	Aquatic	4	74	A5184	Aquatic	3
22	A5243	Dry	2	49	A5202	Unknown	1	75	A5194	Unknown	4
23	A5244	Aquatic	4	50	A5204	Unknown	2	76	A5195	Unknown	1
24	A5245	Dry	4	51	A5205	Aquatic	4	77	A5203	Dry	1
25	A5246	Aquatic	4	52	A5206	Unknown	2	78	A5241	Aquatic	3
26	A5247	Aquatic	4	53	A5207	Aquatic	3	79	A5063	Unknown	4
27	A5248	Aquatic	3								

The first column refers to Fig. 1 and indicates the location where accessions were collected

The second column is the abbreviated accession code

The third column indicates the habitat of each accession

The fourth column indicates how many individuals were used in the experiment

**Fig. 1** Localities of accessions used to test genetic distances among European accessions of *Solanum dulcamara*. Numbers correspond to map nos. listed in Table 1



clustering of single individuals). For a description of the results we focused on the SM dendrogram (Fig. 2).

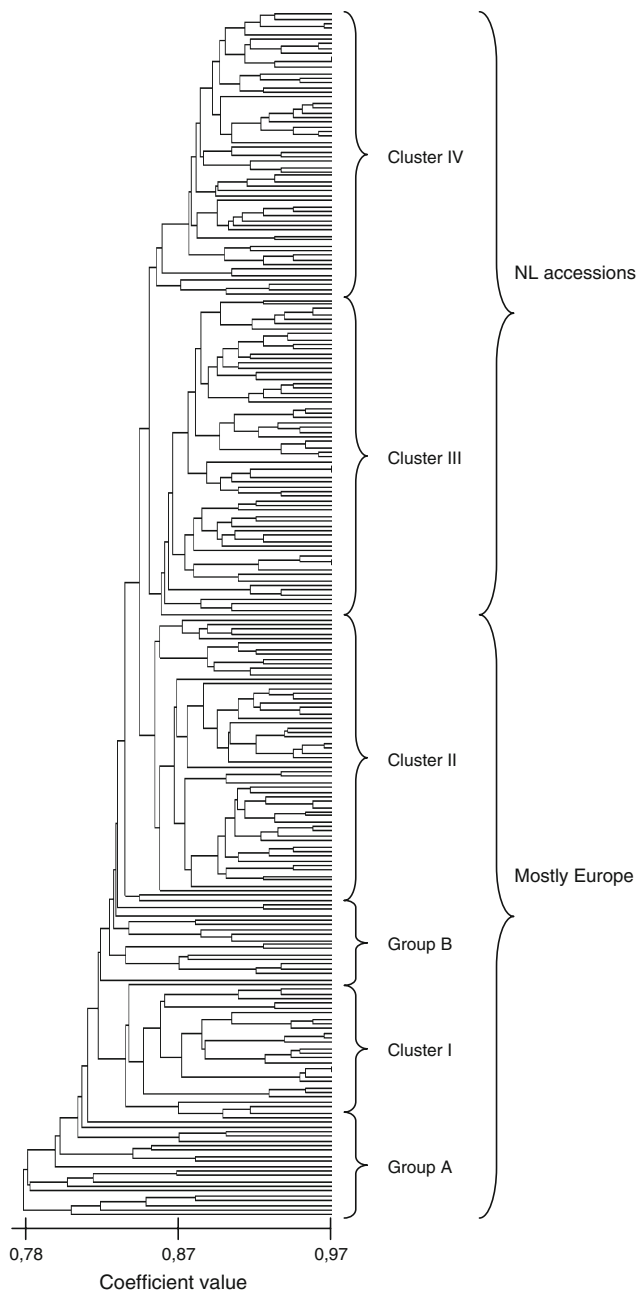
Four major clusters (Fig. 2; clusters I, II, III, and IV) could be recognized, within which several smaller groups were distinguished. There are two groups where no clear structure is present and mixing of individuals from The Netherlands and the rest of Europe occurs (Fig. 2; groups A and B). In these parts of the tree only some individuals interrupt the random pattern by grouping together: one accession from Spain and two from The Netherlands in group B, and individuals from Polish accessions in group A.

Cluster I contains only genotypes collected outside of The Netherlands. This cluster is well structured, and all the individuals from an accession are grouped together (with the exception of accession A5067cGB). The geographical distance and genetic differences between accessions are reflected in their actual position in the dendrogram. Accessions of Italian origin are genetically more similar to the French and German accessions than to accessions originating from North Europe, particularly from Belgium, Great Britain, The Netherlands, and Poland. Genotypes of Dutch and Polish origin are not represented.

Cluster II contains accessions from almost all of Europe. This cluster is divided into three subgroups (1, 2, and 3). The first subgroup (1) contains accessions that were collected outside The Netherlands (with the exception of A5102) and consists of individuals that derived from seeds that had been collected many years before this study was carried out. In this group, clustering of similar genotypes does occur, but is rare and does not reflect the geographical distance between accessions. The second subgroup (2) is restricted to genotypes collected in Poland (with the exception of A5066 and A5202). The third subgroup (3) includes two accessions from The Netherlands (two collected in the Flevoland District area) and two accessions of Italian origin.

Cluster III consists of only Dutch accessions. This cluster shows partial structure, and clustering of individuals from the same accession does occur. Two subgroups contain genotypes collected from the dune areas of western Holland. Also plants collected on the banks of the Nederrijn River (Kesteren) and in Arnhem form a group. However, in both cases, individuals representing those groups are also present in other parts of the dendrogram.

Cluster IV shows high homogeneity among the rest of the Dutch accessions. Clustering of the individuals from



**Fig. 2** Overview of the simple matching (SM) dendrogram based on 288 polymorphic AFLP fragments of European *Solanum dulcamara* accessions, showing the four main clusters (I, II, III, IV) and two groups (A, B) identified in this study. **a**, **b** Enlargements of the four main clusters with the subgroups identified within them. The Netherlands (NL), France (FR), Belgium (BE), Great Britain (GB), Italy (IT), Germany (DE), Poland (PL), Austria (AU), Switzerland (SW), Spain (SP). Individual genotypes are indicated with letters added to the accession code, e.g., A5067c refers to the third individual of accession A5067

the same accession is present, but it is rarely linked with a particular region of sampling. Only accessions from the Wychens Ven area form a separate group, but not all of the genotypes representing this region are clustered.

### Estimation of the breeding system of *S. dulcamara* occurring in natural environments

This experiment was carried out to test whether naturally growing *S. dulcamara* is an out- or in-breeding species. The AFLP results showed that the majority of the offspring contained AFLP fragments that are not present in the mother genotype. In total 24 offspring plants were tested (eight from each parent), and only two plants collected in the region of Egmond aan Zee showed a banding pattern identical to the mother genotype.

### Discussion

The aims of this study were to investigate the genetic structure of European bittersweet and the in- or out-crossing nature of the species. We analyzed accessions collected at various places and habitats throughout Europe to include as much variation as possible. In these experiments AFLP markers were used because of their many advantages for this type of study (Mueller and Wolfenbarger, 1999). AFLP generates a great number of neutral characters, providing high resolution in studies aiming to compare closely related individuals.

The experiment on the genetic distances among accessions of *S. dulcamara* was designed to gain insight into the genetic structure of wild populations, and to see whether cluster analysis reveals any correlation between genomic similarity and geographical provenance of the populations. The average similarity in AFLP patterns of widely sampled bittersweet populations proved to be high (>80%; Fig. 2). Extremely high genetic similarity between populations of wild *Solanum* species was reported earlier for *S. nigrum*. Scholte (2000) detected genetic similarity above 94.7%, but he was dealing with a polyploid and selfing species, where homogeneity is expected. As investigated here, the breeding system of naturally growing *S. dulcamara* plants indicates that it is a cross-pollinating species. We have found only two individuals that could be a result of self-fertilization. However, for *S. dulcamara*, being a diploid out-crossing species, the high similarity found was a surprise. We discuss two possible explanations for the homogeneity encountered. Firstly, bittersweet is a very common plant, at least in The Netherlands. It has an efficient reproduction, both vegetatively and generatively. Also, the seeds are easily dispersed by birds or water. This promotes high gene flow between populations, increasing uniformity. Secondly, in contrast to *S. nigrum*, bittersweet is a perennial plant that can contribute seeds to several generations. In *Populus nigra* L. (Arens et al. 1998) it was found that the longevity of the plant made crosses between generations possible, increasing the similarity within the



Fig. 2 continued

population (82.7%). The same could be the case in *S. dulcamara*.

The genetic similarity between accessions collected outside The Netherlands (Fig. 2; clusters I and II, groups A

and B) is slightly lower than the similarity among the Dutch accessions, reflecting the larger geographical separation of the genotypes used. The similarity among the individuals within cluster I and cluster II accessions is



comparable to that among the material collected in The Netherlands (clusters III and IV).

We detected only partial clustering by habitat or geographical origin of the accessions. The European accessions in cluster I are arranged according to country of origin, but in cluster II, representatives from different countries are mixed, except for Poland. An explanation for this situation might be that those genotypes were derived from various botanical gardens and were propagated for a number of years, during which period uncontrolled crosses could have taken place.

The clustering of individuals originating from the same country as seen in cluster I, and the partial clustering according to habitat or region as seen in clusters III and IV, suggest that there is some level of genetic variation present in the species, but it is only apparent when a few individuals from the separated populations are compared. Differences fade when a large population from the same area is compared (such as for the other Dutch accessions in clusters III and IV). There, the individuals from the same accession cluster, but rarely together, indicating that accessions from local areas are not genetically significantly different from each other.

If individuals from the same region of sampling group together, very often a few individuals from that region are scattered or form small groups in parts of the tree that contain genotypes from other areas. This is the case for the Wychens Ven populations where, in cluster IV, 9 out of 14 individuals are grouped. Three other genotypes of this population are in group B, one individual is in group A, and one is in cluster IV, but separated from the other material from the Wychens Ven. The same is true for the population of western Holland, with a main group (lower part of cluster III) and a small group (upper part of cluster III).

Table 1 lists the habitats of the accessions studied (aquatic, dry, dunes, plains). Within the Dutch accessions, there is a certain clustering of populations originating from dunes, rivers, and the Wychens Ven area. This could indicate that genotypes with certain ecological preferences are more similar to each other. However, Pegtel (1985) studied the germination percentage of two *S. dulcamara* accessions from wet and dry habitats at six constant and five diurnal temperature fluctuations and found little or no evidence of major variability in behavior between the two populations. Still, the differences could be masked by environmental influences. Also, Clough et al. (1979) showed that there was no ecotypic differentiation with respect to light and shade habitats within *S. dulcamara*.

In previous studies authors described morphological variation between *S. dulcamara* populations, e.g., variable density in hairiness of the leaves (Horvath et al. 1977) and erect or procumbent form of growth (van Ooststroom and

Reichgelt 1966), both in natural conditions and in experimental plots. Apparent morphological differences are: hairy or nonhairy stem and leaves, length/breadth ratios and shapes of leaves, differences in the way the inflorescence is formed, and in the number of flowers per inflorescence (own observations). There is also great variation in shape and size of flowers and berries. However, we did not find any correlation between groups of similar phenotypes and groups identified in the AFLP dendrogram. It is possible that these morphological traits are inherited by a relatively low number of genes, thus having little or no influence on the overall structure of the dendrogram. Similar conclusions were drawn by Dehmer and Hammer (2004) who investigated *S. nigrum* and *S. villosum*, finding no correlation between morphology-based and AFLP-based phenograms.

It is concluded that European *S. dulcamara* is a solid, easy-to-recognize out-crossing species that shows a rather faint tendency to subcluster according to geographical origin or ecological niche. The genetic similarity between individuals within and between accessions is high. Apparently, this does not interfere with the species' capacity to adapt to various contrasting habitats such as dry dune areas and wet lands where, at least in The Netherlands, it grows abundantly between common reed, *Phragmites australis* (Cav.) Steud.

Despite the broad occurrence of bittersweet in many European countries and its possible impact as a source of *Phytophthora* in cultivated potato (Platt 1999; Cooke et al. 2002; Flier et al. 2003; Dandurand et al. 2006), our knowledge about this species is very limited. Only a few experiments have been done aimed at answering different research questions (Hare 1983; Wang et al. 1994). Until now, we were lacking insight into the amount of genetic diversity present in this weed species. The present study provides this insight.

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